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Antibacterial Alternatives to a Dying Antibiotic Pipeline

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Abstract

Purpose of review This study summarized the current problems and potential solutions related to the rise of multidrug-resistant bacteria, the lack of antibiotics, and new avenues of research in developing new antimicrobial alternatives, such as using bacteriocins, bacteriophage therapy, antimicrobial peptides, and nanoparticles.
Recent findings Advances in the research of alternative antimicrobial agents in emerging with promising results. These alternative antimicrobials are still developing, and more research is required to bring these products to clinical applications.
Summary A dramatic increase in the emergence of multidrug-resistant bacteria is challenging the research community to find new antimicrobial agents. Multifactorial events have contributed to this emergence, including the lack of research and development of new antibiotics in pharmaceutical companies, the rise of multidrug-resistant bacteria, and the misuse of antibiotics. Another factor exacerbating this problem is that most pharmaceutical companies have closed their antibiotic discovery pipelines. All these factors contributed to the appearance of more resistant pathogenic bacteria, alarming and burdening the health systems.

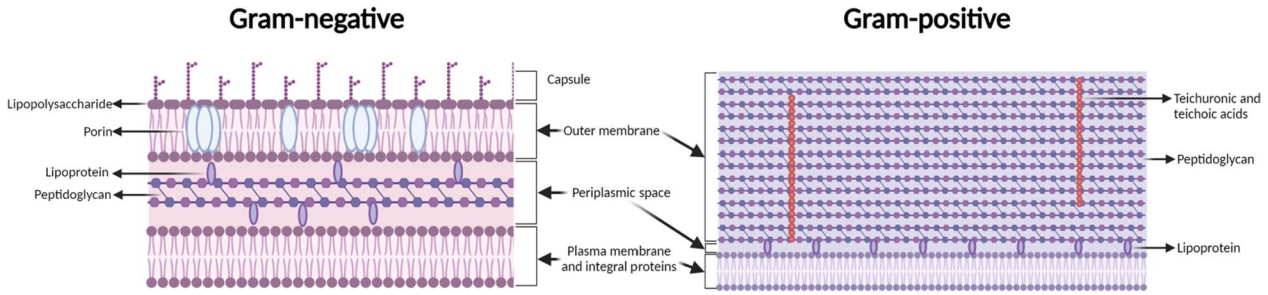

Fig. 1 Cell wall comparison according to Gram staining. Created with BioRender.com (2023) [2••, 3].

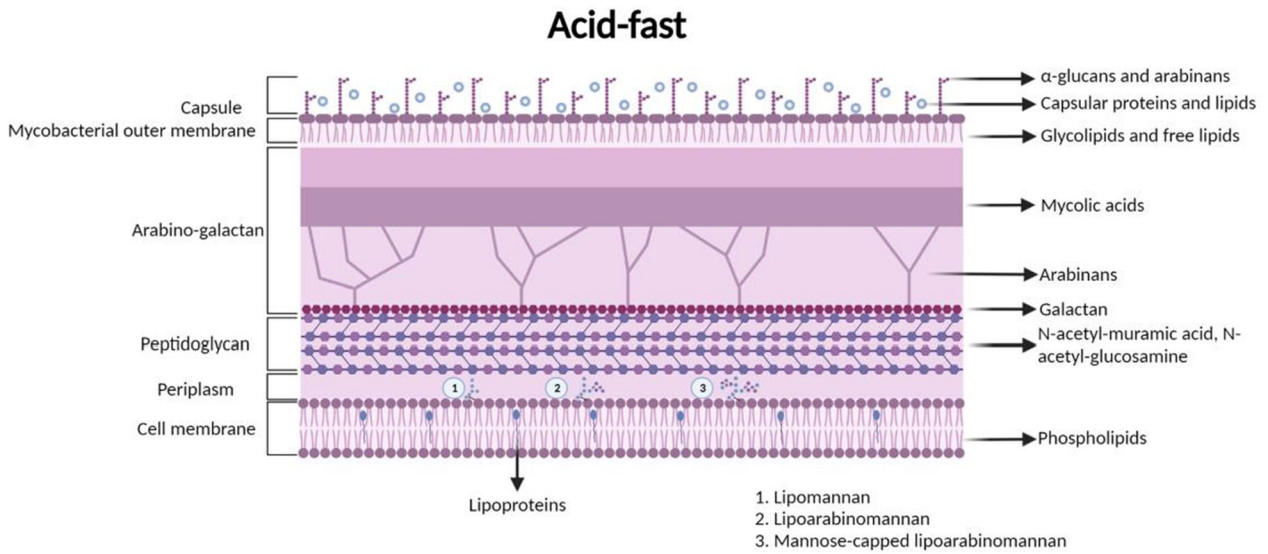

Fig. 2 The cell wall of acid-fast bacteria. Created with BioRender.com (2023) [3].

Bacteria

Bacteria are ubiquitous unicellular organisms able to adapt to environmental changes rapidly. The double time of bacterial cells varies between 20 and 60 min. To visualize bacteria, a microscope is required. However, because of their transparency, their visualization is impaired unless a stain is used. In 1884, the Danish bacteriologist Hans C. Gram published a technique by which bacterial cells can be divided into two groups according to their color after the staining [1]. Based on the staining remnant, bacteria are classified as Gram-positive (purple) and Gram-negative (pink). This separation is based on the ability of the Gram-positive bacteria to retain the dye crystal violet, according to their cell wall composition (Fig. 1).

Not all bacteria are classified in these groups. For instance, mycobacteria species do not respond to Gram staining due to a lipidic cell wall resistant to the stains. However, the Ziehl-Neelsen stain or acid-fast staining was developed, and these species are visualized as a bright red using acid-fast staining. Therefore, mycobacteria are classified as acid-fast bacteria because of their cell wall composition (Fig. 2).

The classification of bacteria in the Gram-positive and Gram-negative groups is important to understand the activity of antibiotics, which will be described later. In this regard, antibiotics can be specific to treat either Gram-positive, Gram-negative, or both, and the broad-spectrum antibiotics.

Viruses

Viruses are ubiquitous infective materials composed of genetic material generally protected by a proteinaceous coat. Only an electron microscope can visualize them. Viruses are obligatory parasites, which require a live cell to multiply. They cannot proliferate outside of the cell because they need the cell machinery to multiply their genetic material and to produce their own proteins depending on the host's machinery. Depending on their structure, viruses could be classified as enveloped or naked (Fig. 3).

Generally, viruses infect by introducing their genetic material into the host cell through different transmission routes. Then, the viral genetic material hijacks the host systems, and the host starts to produce the viral proteins and the genetic material. At the end of the process, the viruses opt to stay inside the host cell or to rupture it and disseminate [5].

To use the host machinery, the genetic material of the virus codes for a few specific proteins able to interact with the host proteins. This is why viruses are very specific to their host and rarely can infect different species.

Antibiotics

Antibiotics are molecules able to inhibit the growth of bacteria. In nature, antibiotics are produced as secondary metabolites by specific groups of bacteria and fungi. The definition of secondary metabolites means that they are not involved in essential metabolic reactions in the cell. Therefore, if the genes responsible for their production are deleted from the bacterial DNA, they can still proliferate. Instead, it might seem like antibiotics are produced to compete for nutritional sources by inhibiting or stopping the development of other bacterial competitors.

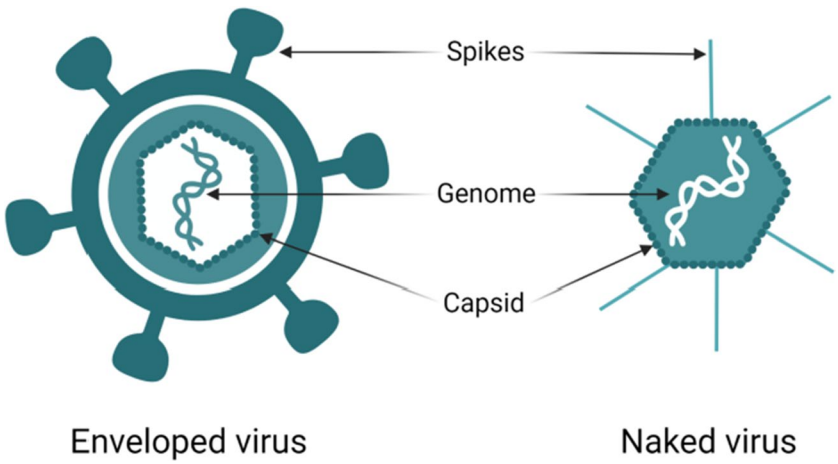

Fig. 3 Example of virus types and their structure Created with BioRender.com (2023) [4].

Penicillin was the first antibiotic discovered in 1928 by Alexander Fleming and started to be used to combat infections in 1942. Since then, new antibiotics have been approved with a concomitant decrease over the last decades (Fig. 4).

When discussing the development of new antibiotic targets, it should be taken into consideration the bacterial target. Many metabolic pathways and enzymes in bacteria are highly conserved across living organisms. Therefore, these pathways and enzymes are not useful as targets because they will inflict similar damage(s) to human cells. Thus, the antibiotic targets should be directed to any bacterial target (for example, protein, biosynthetic pathway) that does not have any similarity in humans. Examples of antibiotics targeting bacteria and resistance mechanisms are depicted in Fig. 5.

Bacterial variation and development of resistance

Bacteria multiply by binary fission, meaning the parental cell divides into two daughters. Each daughter is considered a clone or genetically identical offspring generated by vegetative multiplication. As mentioned, bacteria multiply exponentially (generation time between 20 and 60 min) depending on the species. In a bacterial culture, prolonged growth may generate a residual change because of an adaptive process, resulting in spontaneous mutations even when originating from a single cell. For example, if we calculate the number of mutations (at a rate of 10^{-10} mutations per nucleotide base) in the genome of the bacterium *Staphylococcus aureus*, which contains 2.8 million nucleotide base pairs in its genome, an astonishing number of 300 mutations will be produced in that population within a period of 10 h [11]. For comparison, the human genome will accumulate approximately 60 mutations within 20–25 years [12].

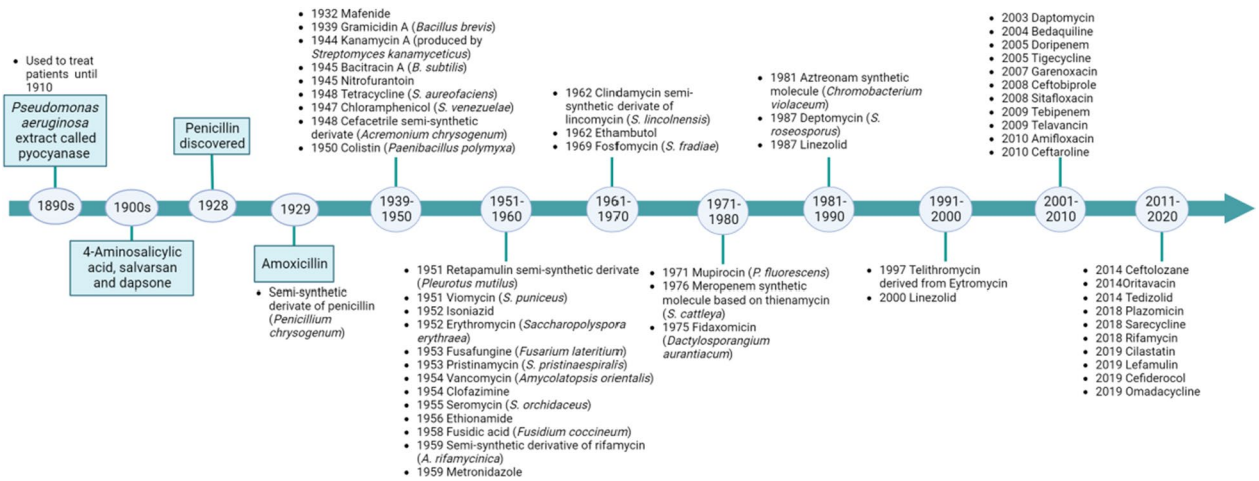

Fig. 4 Antibiotics timeline where the years are references of the first report (to our knowledge) in literature Created with BioRender.com (2023) [6–10].

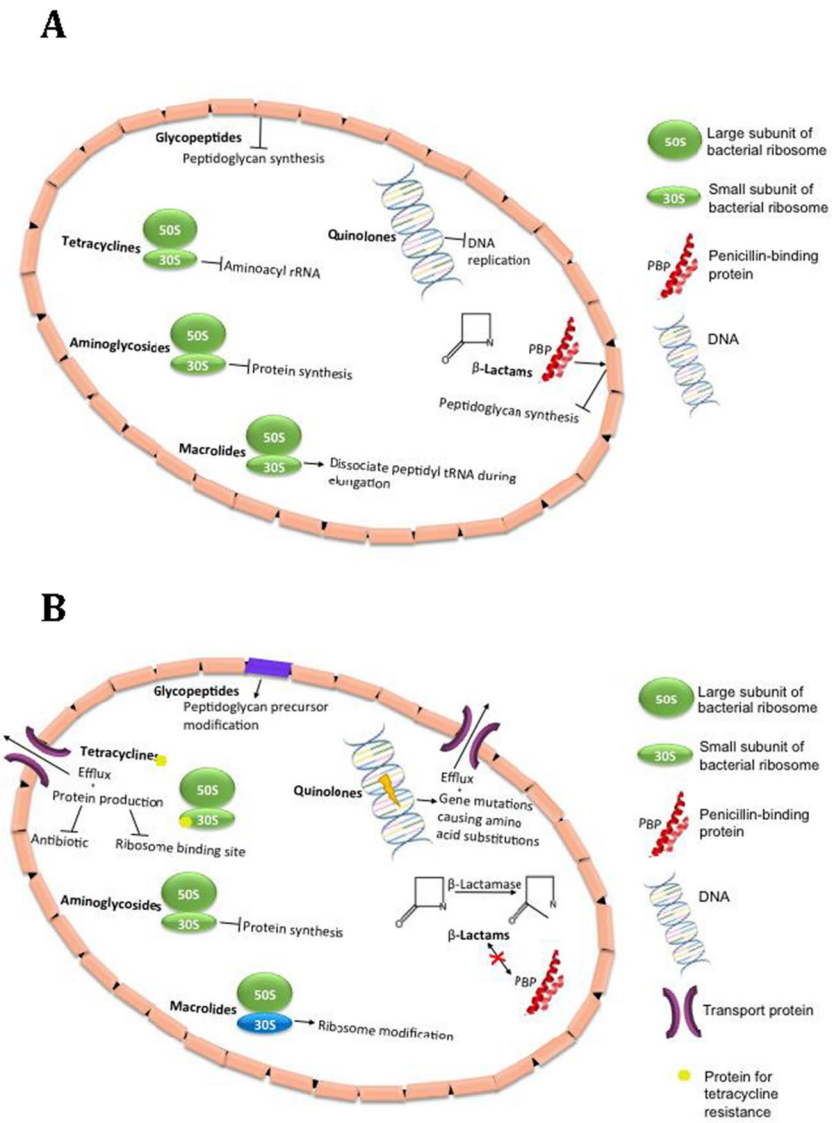

Fig. 5 Mechanisms of bacterial resistance to selected antibiotics. **A** Antibiotic mechanisms. **B** Mechanisms of bacterial resistance [2••].

Drug resistance refers to acquired changes in the bacterial genome against an antibiotic. These genomic changes will continue to exist even when the drug is removed from the environment, and the descendants of this bacterial clone will inherit them. This type of change can be driven either by a change in the sequence of a protein (target of the antibiotic) or by the infection of the bacteria with a foreign piece of DNA that brings new genetic material to it [13].

Treating bacteria with antibiotics produces a selection pressure on the bacterial cells. Based on the information provided above, it is reasonable to think that spontaneous mutations will appear and confer to that specific cell an advantage over the rest of the population. This new resistant strain can

multiply in the presence of the antibiotic because it has developed an adaptive mechanism to cope with the killing activity of the antibiotic. Also, when antibiotics are used for specific times, and then a different class drug is used for another particular time, it is normally called cycling of antibiotics, and it has been proven unsuccessful [13].

This problem is aggravated when bacteria develop resistance to different antibiotics. Therefore, different terms are used depending on the resistance. For example, multidrug-resistant (MDR) bacteria are resistant to at least one antibiotic in three or more antimicrobial categories; extensively drug-resistant (XDR) bacteria are resistant to at least one antibiotic in all but two or fewer antimicrobial categories; and pan-drug-resistant (PDR) bacteria, which are resistant to all antimicrobial categories [14]. Therefore, single or cycling drug withdrawal will not affect the prevalence of the bacteria.

To acquire resistance to an antibiotic, bacteria should develop a mechanism to neutralize it. Bacteria have developed different mechanisms to cope with the presence of antibiotics, which can be generalized as follows: (1) destruction of the antibiotic (enzymatic alteration of the antibiotic molecule by phosphorylation, adenylation, or acetylation), (2) changes in the antibiotic target (mutational alterations in the sequence of the protein targeted by the antibiotic), and (3) reduction in the permeability of the antibiotic (efflux pumps that pump out the internalized antibiotic) [15••].

Once a single bacterial cell generates a mutation, which provides advantages to survive in the presence of the antibiotic, the genetic material conferring this resistance can be transferred to other bacterial cells by small autonomous pieces of DNA, termed plasmids, that are not integrated into the bacterial genome and exist as independent entities. These autonomous pieces are multiplied by the bacteria and transferred to the progeny (vertical transfer) or other species (horizontal transfer) during a process called conjugation [16]. Moreover, bacteria can continually exchange plasmids, and these pieces of DNA may contain resistance genes that will be passed to new bacteria. Interestingly, plasmids can move to new bacteria without an antibiotic, suggesting that resistance can be disseminated in the bacterial population without an antibiotic agent. Some of the main mechanisms of antibiotic resistance are presented in Table 1.

Multidrug-resistant bacteria

The number of deaths related to infections is alarming in Gram-positive and Gram-negative groups, killing nearly 1.27 million people worldwide in 2019, according to the Centers for Disease Control and Prevention (CDC) [18]. The toll of death related to the Gram-positive *Staphylococcus aureus* and *Enterococcus* species is of great threat [19]. According to published studies, *Staphylococcus aureus*-resistant strains, such as MRSA, kill more Americans than HIV infections together with Parkinson's disease and homicides combined [20]. On the other hand, the Gram-negative group with serious infections includes *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* species [19].

Table 1. Main mechanisms of antibiotic resistance. Adapted from [17••]

Mechanism	Examples
Enzyme production	β-lactamases are the most important resistant mechanism among Gram-negative bacteria. This group of enzymes can hydrolyze the β-lactam ring before reaching the penicillin-binding protein target in the cell wall. Aminoglycoside-modifying enzymes can reduce antibacterial activity by diminishing bacterial ribosomal subunit binding
Target site modifications	Ribosomal target site alterations where the erm-encoded rRNA methyltransferases mediate macrolide lincosamide-streptogramin B antibiotics (in *Staphylococcus aureus* and *Enterococcus* spp.)
	Cell wall precursor alterations. In Gram-positive microorganisms, the most common development is the glycopeptide resistance
Reduced antibiotic penetration and accumulation	Porins are important mediators of resistant mechanisms in Gram-negative bacteria. Reflected in downregulations, balance, function, and/or loss in the outer membrane protein channels
	Efflux pumps. Damage in the expression of the efflux pumps in the bacteria. This efflux pumps actively extrude drugs out of the cell
Other mechanisms	Biofilms are structured microbial communities attached to a surface encased in an extracellular matrix, creating a higher tolerance to antimicrobial agents than non-adherent planktonic cells
	Intracellular survival refers to the ability of some species to internalized and survive for long times in the host cells

Healthcare systems cope with antibiotic-resistant infections with a high economic burden. This issue is aggravated because of the invasive procedures performed in these facilities with excessive use of antibiotics to safeguard the lives of critical patients. For instance, a study published in the USA in 2002 revealed that approximately two million people developed hospital-acquired infection yearly, causing 99,000 deaths due to antibacterial-resistant pathogens [21]. The COVID-19 pandemic is also an aggravating factor. As a result, a significant increase in antimicrobial use was observed in health departments and communities in 2020 [22]. The appearance of infections caused in hospitals by antibacterial-resistant pathogens extends the hospitalization of the patients with a subsequent increase in the cost of hospital days, depending on the type of infection [23].

The World Health Organization (WHO) published in 2017 a list of bacterial pathogens with an urgent need for new antibiotics (Table 2) [24]. These priority pathogens and others are commonly known as ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) [17••].

According to a report from the CDC in 2020, among the bacterial strains causing antimicrobial resistance threat, the following species were mentioned: *Clostridioides difficile*, drug-resistant *Neisseria gonorrhoeae*, drug-resistant *Campylobacter*, drug-resistant *Salmonella*, drug-resistant *Salmonella* serotype Typhi, drug-resistant *Shigella*, drug-resistant *Streptococcus pneumoniae*, erythromycin-resistant group A *Streptococcus*, and

Table 2. Priority for research and developing new antibiotics for resistant bacteria pathogens [24]

Priority	Bacterial strain
Critical	*Acinetobacter baumannii*, carbapenem-resistant
	Pseudomonas aeruginosa, carbapenem-resistant
	Enterobacteriaceae, carbapenem-resistant, ESBL-producing
High	*Enterococcus faecium*, vancomycin-resistant
	Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate, and resistant
	Helicobacter pylori, clarithromycin-resistant
	Campylobacter spp., fluoroquinolone-resistant
	Salmonellae, fluoroquinolone-resistant
	Neisseria gonorrhoeae, cephalosporin-resistant, fluoroquinolone-resistant
Medium	*Haemophilus influenzae*, ampicillin-resistant
	Shigella spp., fluoroquinolone-resistant
	Streptococcus pneumoniae, penicillin-non-susceptible

ESBL, extended-spectrum β-lactamase

clindamycin-resistant group B *Streptococcus*. In addition, the CDC also reported an alarming increase in hospitalizations regarding the bacterial strains described in Table 3 [22].

The role of pharmaceutical companies

The role of pharmaceutical companies is to develop drugs to prevent or cure illness. Positive outcomes are based on a continuous introduction of new medicines with an increase in life expectancy to 78 years. However, the drug discovery process is complex, involving billions of dollars with a high-risk

Table 3. Increase in resistant infections starting during hospitalization from 2019 to 2020. Adapted from [22]

Resistant agent	Percentage increase in resistance
Carbapenem-resistant *Acinetobacter*	78
Carbapenem-resistant Enterobacterales	35
ESBL-producing Enterobacterales	32
Vancomycin-resistant *Enterococcus*	14
Multidrug-resistant *P. aeruginosa*	32
Methicillin-resistant *Staphylococcus aureus*	13

investment. Therefore, pharmaceutical companies should carefully assess the profitability of their products before deciding on what drug to invest in.

The process of introducing a new drug in the US market comprises mainly five phases: (1) drug discovery and development (3–4 years), (2) preclinical research, (3) clinical research (4–6 years), (4) US Food and Drug Administration (FDA) review (2–3 years), and (5) FDA post-market safety monitoring [25]. Thus, pharmaceutical companies need to evaluate a long drug discovery process associated with a patent that will expire and a potential drug recall or withdrawal from the market. In conclusion, all the processes involved, from drug discovery to marketing, may last 12–15 years. In the case of introducing new antibiotics, the process is aggravated because of the appearance of bacterial resistance that will reduce the profitability of the antibiotics in the short term. Moreover, when new antibiotics are released, they are often used as a last resort because clinicians prefer to reserve them to treat complex infections. This situation prolongs the antibiotic's shelf life, reducing the company's profitability.

When the pharmaceutical company chooses a specific drug, the transition between the phases is highly risky because regulatory agencies will monitor that each stage is safe for human consumption even before the drug enters clinical trials. For example, selecting a candidate involves screening thousands of compounds; depending on their toxicity, efficacy, or safety, they may proceed to a different step. The investment cost will be recovered only if the candidate drug successfully passes all the phases. As an illustration, 38% of the drugs failed in phase I (safety/blood levels), 60% of the remaining failed in phase II (primary efficacy), 40% of the remaining candidates failed in phase III (considerable, expensive efficacy), and 23% failed to be approved by the FDA [26]. As a result, the number of medicines approved for new treatments has consistently dropped from approximately 35 to 20 new drugs/year in the last decade [27].

Based on all these explanations, it is reasonable to deduce that pharmaceutical companies are more interested in developing new medicines for treating chronic diseases rather than antibiotics. Moreover, patients treated for these chronic diseases will consume the drugs for long (years), even for life, whereas typical antibiotic courses are often days to weeks rather than years. Taking all these concerns together, only a few pharmaceutical companies worldwide continue to develop new antibiotics [28]. Other contributors to the development of new antibiotics, such as the academy, have been affected by funding restrictions [29•]. It might change in the future, as in July 2020, the International Federation of Pharmaceutical Manufacturers and Associations announced an Action fund (AMR) aims to develop 2–4 new antibiotics by 2030, but still more antibiotics development would be needed [30].

Over the last two decades, regulatory agencies such as the FDA have changed how antibiotic clinical trials are executed [31]. For example, using a placebo in the clinical trials of antibiotics is now considered unethical, and instead, trials are addressing the non-inferiority of new antibiotics compared to existing drugs. However, these regulations increase the cost of the trials because larger populations are required, with a concomitant reduction of the profitability [31]. Taking together, changes in the regulations should be pursued to accelerate the approval of new antibiotics [32•]. These changes

can, for example, be based on reducing the clinical trial to a smaller population, which will reduce the cost of the trial, as well as its acceleration for completion.

On the other hand, an examination of the FDA approval during 1998–2003 revealed that the approval of new antibiotics has declined by 56% over the past 20 years [33]. Surprisingly, only 7 of 225 new drugs approved in that period were antibiotics [20], and only two antibiotics had a new mechanism of action [34]. This low number is insufficient to meet the growing needs of our society to cope with infections; therefore, there is a need to find new alternatives to antibiotics.

Patent, exclusivity, and patent cliff

A patent is a property right granted anytime during the development of a drug, whereas exclusivity is the prohibitions and delays on the approval of competitor drugs. Exclusivity time may vary from months to years, depending on the type of exclusivity. Pharmaceutical companies face an additional problem, and it is related to patent expiration. During the discovery phase, after the FDA approval and product launching to the market, companies have a period of approximately 20 years in the USA (from the date the application for the patent was filed) and 10 to 12 years in other countries to recover the investment during the different phases. Once the patent expires, the company faces what is known as a "patent cliff" [35•, 36].

After falling off the "patent cliff," the pharmaceutical company loses the exclusive manufacturing of a specific drug, and it becomes generic; generic medications are sold at considerably lower prices than the original equivalent [37]. Thus, sales and revenues for that specific drug plummet with a price loss of up to 70% shortly after the patent expiration.

Antibiotics, without doubt, have had a positive impact on human health. In the past, deadly untreatable bacterial infections became treatable and stopped to be the leading cause of death. For example, history teaches us that penicillin resistance in *Staphylococcus aureus* appeared in 1945, only 3 years after the onset of its commercialization [38]. Today, *Staphylococcus aureus* has become completely resistant to penicillin and related derivatives [39]. Furthermore, continuing with the same bacterial strain, a rapid increase (5 to 80%) in the antibiotic resistance to ciprofloxacin, thought to be effective because of a novel mechanism unknown in nature, was observed within 1 year of antibiotic use [40].

Physicians routinely prescribe antibiotics to treat infections empirically. For example, treating viral infections with antibiotics does not benefit the patient but instead promotes increasing antibiotic resistance in other bacteria in the patient's microbiota. Thus, increasing the resistance to antibiotics in the normal flora of the patients will neutralize their activity in future infections. For example, after examining a patient with an upper respiratory infection, there is a probability that this infection is caused by viruses, which typically resolve with supportive care rather than direct therapy. Although it may be uncomfortable for a few days,

the infection will resolve without treatment if its origin is viral. To determine the source of the infection, a usual evaluation of bacterial infection requires additional testing, such as a culture, which can come at a higher cost than an antibiotic prescription. Thus, the patient prefers to purchase the antibiotics, knowing viral infection is probable. Under these facts, the patient will press the physician for an antibiotic prescription, making the patient happy. In conclusion, this event, multiplied by thousands of doctor visits/year, develops resistance to untreatable bacteria in the future.

Antibiotic overuse also occurs when physicians administer antibiotics prophylactically when surgeons decide to administer antibiotics to patients facing surgery as a prophylaxis to prevent infections during and after the procedure [41]. According to the CDC, approximately 28% of the antibiotics are prescribed for infections that do not require antibiotics [42]. It is also important for patient to complete entire course of antibiotics prescribed, even if the patient has symptom resolution, to avoid further promotion of resistance to repeat courses of antibiotics [43]. Furthermore, physicians need to prescribe the shortest effective course of antibiotics necessary to treat the patient's infection.

Another aspect of antibiotic overuse is observed in the livestock industry, which uses large quantities of antibiotics to prevent infections [42, 43] and increase animal growth [44]. These infections may take an enormous toll on death quickly and considerably reduce the number of animals, especially in intensive husbandry (like turkey, chicken, and fishponds). Also, these antibiotics reach the environment where they create an ideal niche for developing resistance in the microbiota. Thus, the misuse of antibiotics in these industries puts pressure on bacteria to acquire resistance. An example of this is the presence of resistant bacteria in meat consumers [45]. This phenomenon follows a sequence of events that start with farm antibiotic overuse. This overuse depletes susceptible bacteria and helps with the appearance of antibiotic-resistant bacteria, which are transmitted to humans through the food supply. Moreover, studies have demonstrated that approximately 90% of the antibiotics provided to animals are secreted in urine and stool, which subsequently are used as fertilizers altering the environmental microbiota [45].

Another growing problem is related to antibacterial products found for cleaning or hygienic purposes. For example, their effect on the environment affects the composition of indigenous bacterial populations, directly affecting the development of a proper immune system in humans. This problem grew exponentially with the COVID-19 pandemic. According to the FDA, triclosan was the main ingredient in antibacterial products, significantly impacting the antibiotic-resistant [46].

To tackle antibiotic overuse, national or provincial programs should be established to:

1. Educate health professionals and society to reduce this burden, including behavioral interventions.
2. Develop a rapid test to evaluate whether bacteria or viruses cause an infection.
3. Restrict or limit the excessive use of antibiotics in husbandry by educating farmers and providing governmental oversight.

Alternatives to antibiotics

The fact that the introduction of new antibiotics in the market decreased over the last decades and the appearance of resistance fueled the investigation of alternative sources of antimicrobial agents. These sources would be products different from antibiotics targeting bacteria or their host [47]. The new research proposed venues are presented in Table 4. This review will explain bacteriocins, peptides, nanoparticles, and phages in more detail.

Bacteriocins

Bacteriocins are short or long sequences of amino acids with antibacterial activities produced by lactic bacteria. Their sequences are heterogeneous and classified according to their molecular weight [51]. For example, some consist of short peptide sequences (19–37 amino acids), but others can reach molecular weights of up to 90,000 Da.

Bacteriocins possess antibacterial activity against a broad spectrum of bacteria, making them non-specific and considered safe and natural antimicrobial agents because of their consumption in dairy products since ancient times [52]. In other words, bacteria considered beneficial to humans produce bacteriocins.

Table 4. Recent antibiotics alternatives, their uses, and target. Adapted from [17••, 47–50]

Product	Uses
Bacteriocins	They could also be selective towards specific strains. Resistance to heat and UV. Most of them are generally recognized as safe (GRAS)
Phages	Bacteriophages can be used in small doses because they are able to replicate in the presence of their host bacteria. Wild-type bacteriophages infect and kill bacteria. Engineered bacteriophages have specific therapeutic properties preventing resistance development and rapid elimination. Still currently under review for approval by the FDA. They could also be selective towards specific strains
Nanoparticles	They target multiple biomolecules concurrently; therefore, it is difficult for bacteria to develop resistance against them
Antibodies	They bind and inactivate pathogens, and it is a bacterial-specified therapy. Do not damage the microflora
Probiotics	Live microorganisms, which when administer in specific amounts, may have a beneficial health effect in the host. Easy availability (dairy products). Mainly targeting *Clostridium difficile*
Lysins	Enzymes that are produced by bacteriophages to destroy the cell wall of bacteria. They are also able to weaken biofilms produced by bacteria. Not prone to resistance development. Mainly targeting Gram-positive bacteria
Vaccines	Reduction of infections, but more research and investment of new target vaccines are needed
Peptides	Rapid action, low target-based resistance, and low immunogenicity. Not prone to resistance development
Liposomes	Acting as decoys for toxin (secreted by pathogens) to reduce damage to mammalian cells
CRISPR/Cas9	Specificity towards specific strains and reversal of antibiotic usage. Easy to synthetize, but are still under toxicity testing. Not prone to resistance development. Broad-spectrum activity is an advantage
SMAMPs	

Lactic bacteria produce bacteriocins in the intestine probably to gain access to nutrients in a highly competitive environment with trillions of different bacterial species striving to survive. However, bacteriocins are not exclusive to the lactic bacteria group. Other bacterial strains have been shown to produce bacteriocins, such as *Fusobacterium mortiferum* and *Enterococcus faecium*, which were isolated from chicken with in vitro antibacterial activities [53, 54].

Bacteriocins are grouped into different classes, but lantibiotics and thiopeptides are the most extensively studied [55]. For example, lantibiotics effectively control Gram-positive infections in vitro and in vivo caused by *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumonia*, and *Streptococcus pyogenes* [56–59].

On the other hand, thiopeptides have shown extraordinary results as antimicrobial agents, but their applications have been restricted because of water solubility issues [60, 61]. However, analogs of these thiopeptides have been generated with successful applications to control infections of *Clostridium difficile*, *Salmonella enterica*, and *Staphylococcus aureus* using rodent models [62–64].

Although bacteriocins can be delivered as bacteriocin-producing bacteria, their activity in the intestinal tract should be monitored. In the case of bacteriocin treatment in chicken, it has been shown that low molecular weight bacteriocins are active in the intestinal environment. For instance, the secretion of curvacin produced by *Lactobacillus curvatus* showed growth inhibition of *Escherichia coli* and *Listeria innocua* in the digestive tract [65]. Furthermore, experiments performed to determine the degradation of the bacteriocin in the digestive tract revealed that it was degraded in the ileum portion of the intestine [65].

The bacteriocin nisin produced by *Lactobacillus lactis* showed a change in the fermentation parameters in an artificial rumen model [66]. These changes are probably attributed to changes in the microbiota of the rumen caused by the bacteriocin administration.

Mechanism of action of bacteriocins

Studies have reported that bacteriocins target different pathways. For example, nisin, lantibiotics, and other bacteriocins (class I) bind the lipid II, an intermediate in the peptidoglycan biosynthesis, specifically in Gram-positive bacteria [67–70]. Moreover, upon binding lipid II, lantibiotics and lactococcin A (class II) enable the formation of pores in the bacterial cell membrane leading to a membrane potential unbalance, resulting in cell death (Fig. 6) [67, 68, 70].

Pore formation is a mechanism observed in different types of bacteriocins. Their activity depends on binding specific receptors on the bacterial membrane to exert their action. For instance, some bacteriocins recognize the cell envelope-associated mannose phosphotransferase system (Man-PTS), whereas others recognize the iron-binding siderophore receptors (e.g., FepA, CirA or Fiu) [71, 72].

Other mechanisms of action of bacteriocins, such as the interference in gene expression and protein biosynthesis, have been proposed. Examples

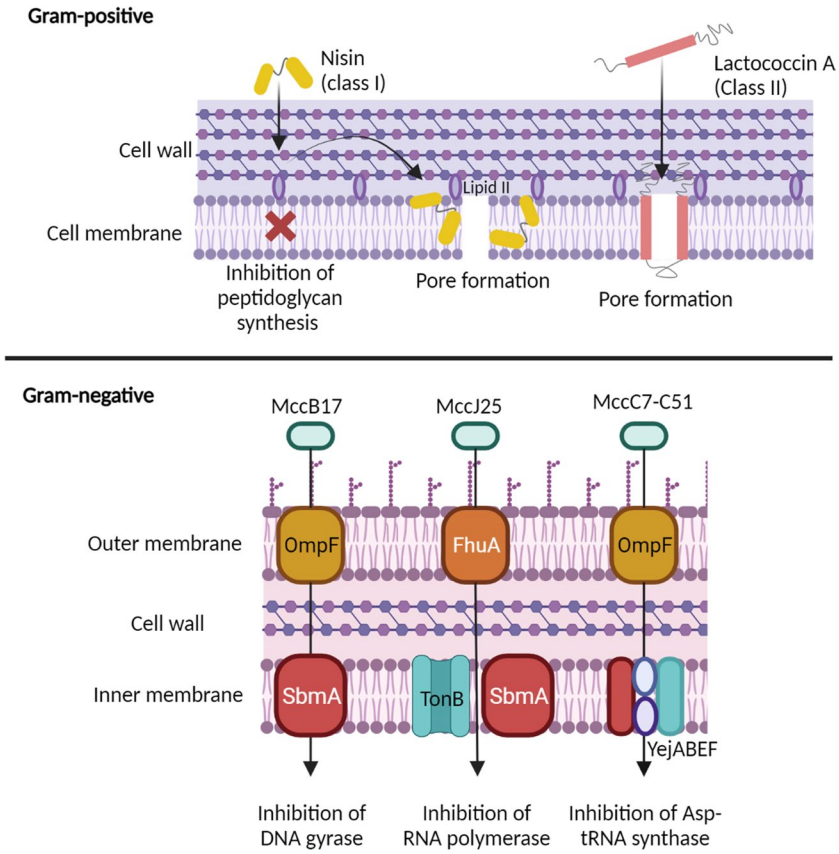

Fig. 6 Bacteriocins mode of action in Gram-positive and Gram-negative bacteria. Created with BioRender.com (2023) [70].

include interference with DNA (e.g., inhibition of supercoiling mediated by gyrase), RNA (e.g., blocking mRNA synthesis and binding to the 50S ribosomal subunit), and protein synthesis (e.g., modification of amino acids and binding to the elongation factor Tu) [73–77]. For instance, microcin J25 (MccJ25) can inhibit RNA polymerase, MccB17 inhibits DNA gyrase, and MccC7-C51 inhibits aspartyl-tRNA synthetase in Gram-negative bacteria (Fig. 6). In addition, bacteriocins (such as MccE429) in Gram-negative bacteria have also been reported as pore formation products [70].

Mechanisms of resistance to bacteriocins

The appearance of resistance is always a concern that may be developed because of changes in the membrane composition/structure. In this regard, resistance to nisin has been reported in specific strains of *Clostridium* and *Listeria* [78–80]. Moreover, exposure of the bacteriocins microcin-24 and nisin to *Salmonella enterica* and *Streptococcus bovis*, respectively, showed that the resistant cells had also resistance to other antibiotics [81, 82]. Target bacteria can also evade bacteriocins by neutralizing the negative net charge of the cell wall [83].

Resistance mechanisms have been mainly identified with bacteriocins targeting the cell envelope. For example, it has been shown that a decrease in the bacteriocin receptor targeting the lipid II conferred resistance to *Staphylococcus aureus* [69] and a regulation of the ABC transporter in *Listeria monocytogenes* [84]. However, mutations on genes encoding the RNA polymerase subunit and the gyrase have also been observed [85–87]. Other resistant bacteria against bacteriocins are *Enterococcus faecalis* (resistant to pediocin, nisin, divercin V41, and lacticin 3147) and *Enterobacter faecium* (resistant to mundticin KS) [83].

Resistance to bacteriocins has already been reported, and potential solutions should be considered to reduce the appearance of such resistance. These include the derivatization of the original molecule to synthesize new molecules that may bind the receptors to reduce their recognition by the bacteria [88]. Alternatively, a cocktail of bacteriocins combined with other antibacterial agents should also be evaluated.

Bacteriocin delivery

One attractive system for delivering bacteriocins is the use of *Lactobacillus* strains. For example, *Listeria monocytogenes* and enterohaemorrhagic *Escherichia coli* growth inhibition in a mouse model have been reported using *Lactobacillus casei* strain LAFTI L26 [89, 90]. Interestingly, bacteriocins successfully controlled buccal pathogens using an engineered *Streptococcus mutans* strain, producing the bacteriocin mutacin 1140. This bacteriocin controlled plaque formation [91], and the engineered strain was retained in the buccal microbiota for 14 years after the application [92].

Antimicrobial peptides

Small antimicrobial peptides are produced by probably every organism to cope with bacterial invasion. Antimicrobial peptides are short peptides with a molecular mass of 1000–5000 Da. Analysis of their sequences revealed that they interact with the negatively charged bacterial membranes based on their net positive charge [93]. Further analysis of antibacterial peptides revealed that in their sequences, a hydrophobic sequence is required to bind to the bacterial membrane and a conformation change to intercalate in the membrane.

Structural analysis of the peptides showed that they might acquire different 3D conformations, such as helices, sheets, or loops [94]. The structure of the peptides is fundamental because a re-design of the secondary structures of the peptides may increase their antibacterial activities or their stability, being more resistant to the action of proteases [95–98].

Mechanism of action of antibacterial peptides

The main mechanism of action of antibacterial peptides is permeabilization. Therefore, they depend on the interaction with the cell membrane. This

interaction involves an electrostatic interaction when the cationic peptide binds to the negatively charged outer bacterial envelope. The negative charge on the cell membrane results from phosphate or lipoteichoic groups present in the lipopolysaccharides or surface of Gram-negative and Gram-positive bacteria, respectively. Once the electrostatic interaction occurs, hydrophobic interactions allow the insertion of them into the outer membrane structure in Gram-negative strains (Fig. 7) [99, 101].

There are four main models of pore formation in bacterial membranes regarding the antibacterial mechanism of peptides (Fig. 7), barrel-stave model, toroidal pore model, Capet model (which is like the toroidal pore model, but in the carpet model, this kind of pore formation has more than one presence in the membrane), and aggregate model [99].

Antibacterial peptides act in different targets, such as inhibiting nucleic acid and protein synthesis, enzymatic activity, and cell wall synthesis [102]. For example, buforin II (isolated from a frog) penetrates the bacterial membrane and binds both DNA and RNA molecules in the cytoplasm of *Escherichia coli* [103]. Likewise, other peptides inhibit DNA and RNA synthesis without destabilizing the bacterial membrane [104–106] or protein synthesis [105, 106]. Other inhibitions include the enzymatic activity of pyrrhocidin that inhibits the activity of the heat shock protein DnaK (ATPase activity for a correct folding) and the transglycosylation of lipid II for peptidoglycan synthesis has been reported [107–109].

Mechanisms of resistance to antibacterial peptides

As bacteriocins, the development of resistance against antimicrobial peptides has been shown. Studies have shown that specific genes can confer increased resistance to antimicrobial peptides, such as the gene *rcp* in *Legionella pneumophila* [110, 111]. The suggested reason for resistance to peptides of *Enterococcus faecalis* and *Listeria monocytogenes* is the change in membrane fatty acid composition, increasing the contents of D-lysine in the wall teichoic acid and L-lysine in the membrane phospholipids [83]. Other resistance

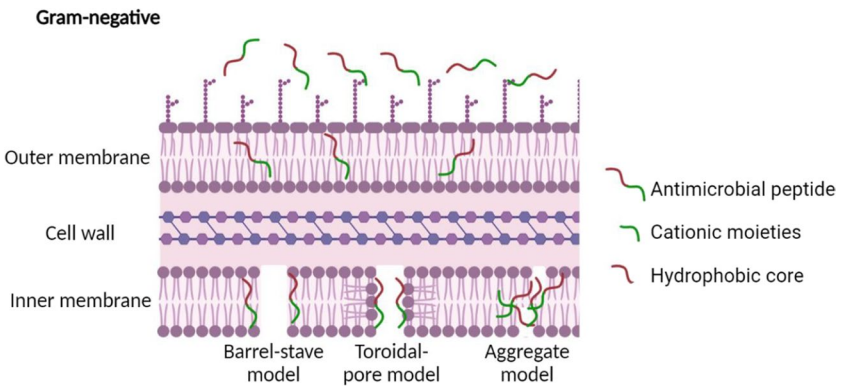

Fig. 7 Main models of pore formation in Gram-negative bacteria regarding the antibacterial mechanism of peptides. Created with BioRender.com (2023) [99, 100]

mechanisms have not yet been elucidated, and whether this resistance is transferred between bacteria.

Antimicrobial peptides, such as gallinaceans, have been isolated from leukocytes in chicken and showed antimicrobial activity against *Listeria monocytogenes*, *Escherichia coli*, and the yeast *Candida albicans* [112]. Other antimicrobial peptides were isolated from turkey and showed activity against *Staphylococcus aureus* and *Escherichia coli* [113].

The use of antimicrobial peptides faces stability issues. As a result of their proteinaceous nature, they are subjected to degradation by proteolytic enzymes highly abundant in the body. Although the immune system also produces antimicrobial peptides, they do not face any vulnerability as their activity is very close to the production site. Thus, the potential use of these antimicrobials should address the proteolysis issue, designing more resistant peptides, including chemical modification and encapsulation to protect them or to develop a slow-release system. In addition, other delivery alternatives have been proposed, such as their production in genetic-modified plants, which can be used as animal feed [114].

Antimicrobial peptide delivery

Antimicrobial peptides are quickly degraded, decreasing their antimicrobial properties and short time of effectiveness. Therefore, nanocarriers are an excellent option to improve their stability. For example, a study demonstrated *Escherichia coli* and *Staphylococcus aureus* death when exposed to antimicrobial peptides encapsulated in chitosan nanoparticles (NPs) [115].

Peptides can be conjugated on metallic or mesoporous silica NP surfaces to be directly available to interact with bacterial cell membranes to achieve antimicrobial effects. However, metallic and mesoporous NPs could also have some inherent antimicrobial properties. Lipid NPs, such as liposomes, do not have any intrinsic antimicrobial properties, but they are effective carriers of antimicrobial peptides. Other nanostructures, such as dendrimers (which do not have intrinsic antimicrobial properties), can be formed using antimicrobial peptides to induce antimicrobial effects [116].

Nanoparticles

Using NPs to control bacterial diseases has shown promising results. Over the last decade, NPs mainly synthesized from Ag, Au, Zn, and Cu have been tested as potential antibacterial agents. NPs possess a range between 1 and 100 nm and have different physicochemical characteristics than the bulk material. One of their characteristics is the large surface area compared to their volume, making them very reactive.

AgNPs are the most studied NPs probably because of the prolonged use of Ag in medicine already described in the ancient literature by Hippocrates of Kos (c.460-c.370 BC). As a result of the enormous number of papers published regarding AgNPs as antibacterial agents, this section will focus only on these NPs. During the process of AgNP synthesis, Ag ion (Ag^+) is reduced to

Ag^0 by using chemical reductants. However, over the last years, a more friendly technology using plant extracts has been proposed to diminish the toxicity problems linked to classical chemical synthesis [117, 118].

Physical characterization of the AgNPs revealed that the shape and size are essential parameters that profoundly affect their antibacterial activity. For example, maximal activity was achieved when the size of the AgNPs is <40 nm, and the highest activity was measured when an elongated or spherical shape was attained [2••, 119–121].

Activity mechanisms of AgNPs

The antibacterial activity of AgNPs is based on different mechanisms. It is unclear whether AgNPs internalize into the bacterial cell or, due to their activity, the membrane ruptures allowing their internalization [2••, 122]. Many studies indicated that the adsorption of the NPs on the extracellular portion of the bacteria is the main mechanism of toxicity [119]. As a result of the adsorption, depolarization of the cell wall ensues, and the cell becomes more permeable, leading to cell death [123, 124]. Other studies have reported that AgNPs aggregate on the bacterial cell wall, causing a cell envelope disruption [119, 125, 126] with interactions with different functional groups, such as carboxyl, amino, and phosphate groups, leading to Ag precipitation [127].

Another mechanism of bacterial toxicity is generating reactive oxygen species (ROS) by the AgNPs. ROS (free radicals, superoxides, and peroxides) are generated in any cell due to the metabolic pathways (Fig. 8); however, cells have evolved different systems to cope with the toxicity of these ROS. The production of ROS, either intracellular or extracellular, may lead to membrane disruption [128], including lipid peroxidation [129].

Other toxicity mechanisms are related to inhibiting the bacterial respiration [130–132] and protein and thiol binding (109, 114, 119). Notably, the amino acid cysteine has a high affinity for Ag^+. Therefore, the complexation between cysteine and Ag^+ affects the proper folding of proteins and many

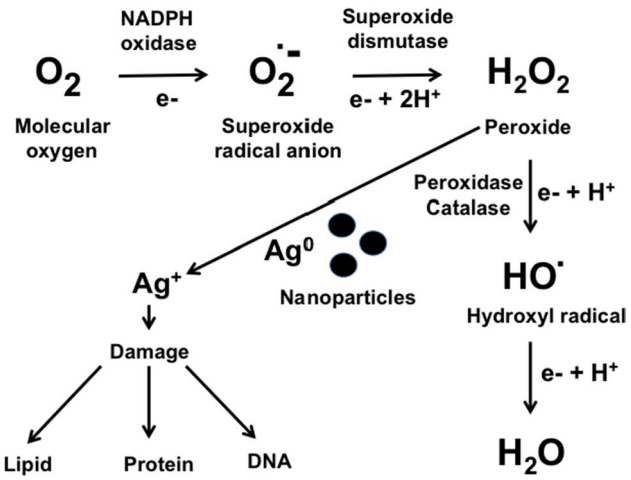

Fig. 8 Production of ROS and their activity in the AgNPs [2••].

enzymes' catalytic activity. Then, AgNPs target a diversity of enzymes simultaneously with detrimental effects on the bacterial cell (119, 120). A model of AgNP toxicity in *Escherichia coli* is depicted in Fig. 9.

Mechanisms of resistance to nanoparticles

When bacteria are exposed to low concentrations of AgNPs, stress is caused to the cell, which could stimulate resistance in the bacteria. The research of resistant bacteria to AgNPs is still in its infancy, and the question remains whether the resistance is driven by the AgNPs (the released Ag^+) or a combination of both and other factors. Interestingly, a study has shown resistance to AgNPs in *Escherichia coli* K-12 MG1655 but not to the Ag^+ [133]. Research is still needed better to understand the resistant mechanisms of bacteria towards NPs.

Bacteriophages

Bacteriophages or phages are viruses that infect and multiply in bacteria [134]. As mentioned earlier, viruses infecting cells can be released into the environment by bacterial cell destruction or lysis. Phages are attractive for therapy because of their interaction specificity with only a specific strain of bacteria. Phages interact with their hosts by identifying specific binding sites (bacterial receptors), rendering other strains that lack these receptors unaffected. On the other hand, this host specificity may signify a challenge for phage therapy. For example, lytic phages that infect all *Salmonella* serovars (the same species but with differences in the surface antigens) have yet to be discovered.

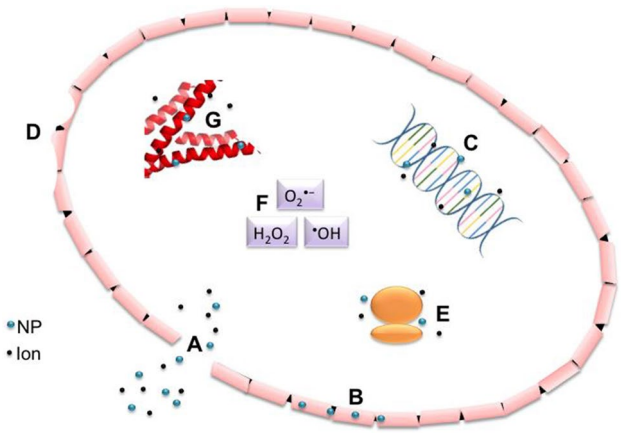

Fig. 9 A model showing the toxicity of AgNPs in *Escherichia coli*. (**A**) Disruption and disintegration of the membrane/cell wall. (**B**) AgNPs access the periplasmic space gaining entrance to the cytosol where they interact with (**C**) DNA and (**E**) ribosomes (protein synthesis impaired), generating (**F**) ROS and (**G**) binding to cysteines in proteins (2).

Though historical interest in phage therapy was limited, the appearance of antibiotic resistance in bacteria fueled the re-emergence of phage therapy as a viable treatment option. Though promising, therapeutic phages face issues related to interaction with the target bacteria. Introducing the viral genetic material can cause undesired changes in the bacterial strain. For example, some phages may integrate into the bacterial chromosome, introducing new characteristics or modifying the expression of host genetic characteristics. These characteristics may include effects on the secretion of bacterial virulence factors, such as toxins or antibiotic-resistance genes [135–138]. Therefore, phages are desired to enter a lytic cycle to destroy their bacterial host rather than be incorporated into the bacterial chromosome. Thus, cell lysis is preferred in phage therapy because of the destruction of the host, reducing the chances for viral interactions with the bacterial chromosome.

Future phage therapy will focus principally on the digestive and respiratory tracts with little possibility of being used as systemic therapy. In blood, phages will be exposed to circulating antibodies, which will clear the phage from the blood circulation. However, in the digestive tract, phages are subjected to adverse factors such as pH changes, which might change their antimicrobial activity. For example, the load of *Salmonella enteritidis* was reduced on contaminated melons but not in apple slices with a pH of 4.2 [139].

Safety concerns have also been elevated in the production of phages for phage therapy. For example, phages should be produced in live microorganisms, and their production is limited to their pathogen hosts. In this regard, phages can carry genetic material from the host that, in this case, is the pathogen and transmit it to other bacteria. This scenario is not a frequent event, but producing the phages in a non-virulent pathogen will be desirable to reduce this likelihood. In some applications, using the enzyme responsible for the lysis of the host may suffice to control the pathogen [140]. Still, it may be limited to topical applications or mucosal infections to avoid traveling through the digestive tract, with little possibility of survival.

Although disadvantages related to phage therapy have been discussed above, it is still considered a natural alternative to control infections in humans [141, 142]. Furthermore, its use is supported by studies that showed protective effects in different animal models. For example, intramuscular injection of phage-protected mice infected with *Escherichia coli* O18:K1:H7 and a reduction in the enteropathogenic *Escherichia coli* strain was measured in the digestive tract of infected calves, piglets, and lambs treated with phage therapy [143, 144]. Similar studies showed the effectiveness of phage therapy when mice were infected with a vancomycin-resistant *Enterococcus faecium* infection [145].

An alternative approach based on a genetically engineered phage to deliver genetic material. The approach uses lysogenic (non-lytic) phage to deliver the genetic material, which encodes proteins with bactericidal activity, such as toxins [146].

Phage therapies against MDR bacteria summarized in [147] have shown relative success in animal models, although the models do not represent human infections. These studies are designed to inoculate high and lethal animal pathogen doses, which cause rapid death. Also, the phage treatment commences after the bacterial inoculation, limiting the effectiveness of the therapy. In some

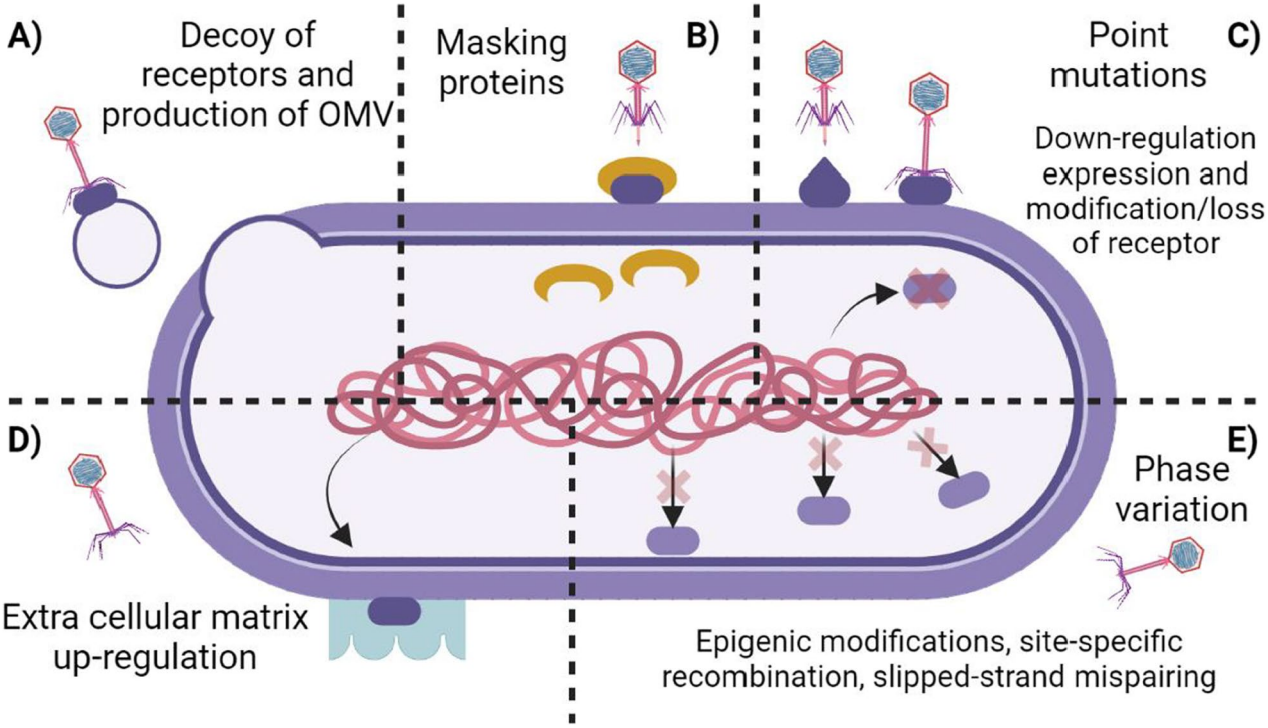

Fig. 10 Phage resistance to bacteria. (A) Outer membrane vesicles prevent the phages from encountering the bacteria. (B) Receptor masking proteins in the bacteria make phages unavailable. (C) The point mutations lead to the downregulation of their expression or the modification or loss of phage receptors. (D) Increase in extracellular matrix production, which hides the phage receptors. (E) Three mechanisms make phage variation: epigenetic modifications, site-specific recombination, and slipped-strand mispairing. Created with BioRender.com (2023) [157].

specific diseases, such as cystic fibrosis, where phages could provide an alternative therapy, no mouse model represents the phenotype of mucus clogs in the airways and lungs. Other factors include the animal physiology different from that of humans, anatomical differences, intestinal pH, and microbiota [148]. However, studies have been conducted to evaluate the safety of phage therapy. For example, no adverse effects were observed when a phage cocktail was assessed against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* in venous leg ulcers [149]. In a randomized, double-blind study, chronic otitis caused by *Pseudomonas aeruginosa* was treated with a phage cocktail. Results showed a significant reduction of the bacterial load with no adverse effect [150]. Another clinical trial aimed to treat burn wounds infected with *Pseudomonas aeruginosa* using a cocktail of 12 phages [151] showed no significant differences compared to traditional therapies. The results of these trials indicate that many challenges are still pending to be resolved prior to the acceptance in clinical applications.

Mechanisms of resistance to phages

The main mechanism of bacterial resistance to phages is related to phage receptors in the bacteria. Therefore, the bacteria can change, hide, and lose phage

receptors. Usually, the response mechanisms start when there is a stimulus. For example, when there is a change in the bacteria cell wall, as in *Bordetella* spp. and *Shigella flexneri*, there is also a loss of phage receptors. Other bacteria, like *Pseudomonas* spp. and *Enterobacteriaceae*, can secrete polymeric substances (EPS) like glycoconjugates to avoid the adhesion of the phages to the bacteria [152, 153].

Another resistance mechanism to phages is viral DNA removal, which could be achieved through different methods. The equivalent of the immune system in bacteria is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), which protects genetic information from possible viral or plasmid attacks. However, the proteins in the superinfection exclusion system (Sie, based on membrane-associated proteins) interact with some DNA injection proteins when there is a binding between a phage and a membrane receptor of the bacteria. Therefore, the DNA injection is stopped preventing the infection from spreading [152]. To avoid resistance to phages, phage cocktails could be a good alternative. Using different phages targeting several receptors, each with a diverse genetic clade could increase the chances of mitigation against absorption loss or generic protection mechanisms from the host [153]. For example, studies have reported that using a cocktail of lytic phages effectively controlled *Salmonella* isolates in chicken and fresh-cut fruits [139, 154–157]. Host adaptation leading to phase resistance is also shown in Fig. 10 [157].

Conclusions

The continued misuse of antibiotics and other agents has accelerated the appearance of bacteria showing multidrug resistance. The lack of new antibiotics introduced by pharmaceutical companies has aggravated the problem, especially after the COVID-19 pandemic. Both situations have led to a dangerous position for humanity, which will need to cope with a lack of antibiotics to combat diseases in the short term. To overcome this problem, the scientific community has started the development of new antibacterial agents. Therefore, it is of great importance that everyone in our society takes responsibility for reducing the burden of diseases, including regulatory agencies by accelerating the process of approvals, governmental agencies to provide incentives to pharmaceutical companies to continue with the development of new antibacterial agents, agricultural extension to educate the farmers for wise use of antibiotics, and public advisory to be aware of the misuse of antibiotics.

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Declarations

Conflict of Interest
The authors declare no competing interests.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by the authors.

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