Fighting Antimicrobial Resistance

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EDITORIAL FOREWORD

When antimicrobials were widely introduced in the 1940s they were considered as “miracle drugs” because they could successfully treat a broad range of common infections as well as more difficult bacterial diseases.

Since then, this medical innovation has saved millions upon millions of human lives around the globe. Due to overuse or misuse of antimicrobials, some bacterial strains have developed resistance to different antimicrobial drugs, although the antimicrobial resistance (AMR) is a natural process, some degree is expected to develop against all antimicrobials, even when treatments are optimal. It occurs when microorganisms evolve to be able to resist the medicine that has been used to combat them. Resistant microorganisms can survive or even grow in the presence of a concentration of antimicrobial that is usually sufficient to inhibit or kill non-resistant microorganisms of the same species.

Medical doctors and scientists are alarmed that if the trend continues, we may enter a “post-antibiotic era” when even minor infections can prove fatal.

Antibiotics are among the most commonly prescribed drugs used in human medicine, but up to 50% of the time antibiotics are not prescribed properly (often given when not needed or with incorrect dosing or duration).

Treatment failure caused by AMR contributes to: additional side effects; longer hospital stays; psychological disorders due to reduced quality of life; burden on families; and a greater likelihood of death as a result of inadequate or delayed treatment. AMR also affects patients who are not infected with resistant organisms.

Infections caused by resistant organisms currently claim at least 50,000 lives each year across Europe and the USA, and hundreds of thousands of deaths are being caused in other areas of the world. AMR may be the greatest challenge to face health care in the 21st century.

While the development of AMR has been accelerating, the development of new antimicrobial agents has slowed substantially in past decades. For example, the ageing of the USA population has shifted medicine discovery
efforts towards agents for chronic medical conditions that are more prevalent among the elderly, such as hypercholesterolaemia, hypertension, mood disorders, dementia, arthritis and cancer.

Factors that will largely determine the future extent of AMR are: pathogen and microbial ecology; prescribing and dispensing practices; population characteristics; and health care policy.

Activities implemented in many countries contribute to AMR containment through increased capacities for improved infection prevention and control, stronger AMR stewardship, and the establishment of regulatory systems, national action plans, standard treatment guidelines, essential medicines lists, and updated pre-service curricula. It is important to build capacity, detection systems, and laboratories to strengthen and improve medicine use, improve infection prevention and control practices, and detect and report priority AMR pathogens.

The emergence of AMR is a complex problem driven by many interconnected factors, in particular the overuse and misuse of antimicrobials.

The aim of this book is to provide some perspective on this very important subject and to provide cutting-edge knowledge and reviews of the activities and various aspects of antimicrobial resistance containment.
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Chapter

1

FIGHTING ANTIMICROBIAL RESISTANCE IN ESKAPE PATHOGENS

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Chapter 1

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1.1. INTRODUCTION

Antimicrobial resistance (AMR) is the ability of a microorganism to withstand antimicrobial compounds. The evolution of AMR is a natural phenomenon that results from bacterial gene mutations or the acquisition of exogenous resistance genes present in mobile genetic elements that are able to propagate horizontally between bacteria. The use of antimicrobial agents and the transmission of antimicrobial-resistant microorganisms are the most important factors that lead to the emergence and expansion of AMR [1].

Bacteria can acquire several mechanisms that make them resistant to various families of antibiotics. This can have severe consequences when the suitable antibiotic treatment to fight the infection is lacking [1].

AMR problems require collective efforts at the country level, as well as close international teamwork. In Europe, the European Antimicrobial Resistance Surveillance Network is the prime system for monitoring AMR in bacteria that are etiological agents of serious infections [1]. The European Centre for Disease Prevention and Control (ECDC) reported that resistance to multiple antibiotics is an increasing worry in the EU and stated that 'With increasing resistance even to last-line antibiotics we face a frightening future where routine surgery, childbirth, pneumonia and even skin infections could once again become life-threatening' [2].

The Centers for Disease Control and Prevention (CDC) reported that at least 2 million people are infected with a resistant microorganism every year in the United States which results in at least 23,000 individuals dying every year [3].

Multidrug resistance is one of the three principal concerns to global public health [4]. Several factors are responsible for this situation, such as an increase in the global use of antibiotics [5], the absence of widely-used best practices in the management and training of antibiotic administration [5,6], the inappropriate use of antibiotics (i.e. insufficient dosage and prescriptions to treat mild bacterial or viral infections) [7] and the extensive and lawless use of antibiotics in animals to enhance meat production [8]. Another significant factor in the rise of antibiotic resistance is the propagation of resistant strains among the population or from other environmental sources [3]. Finally, a lack of sufficient knowledge about the mechanisms involved in bacterial tolerance and persistence is associated with AMR [9-11].

In 2016, the World Health Organization (WHO) generated a priority list of pathogens with the principal aims of allocating funding to facilitate the global coordination of research and promoting strategies to identify new active antiinfective agents against multidrug-resistant (MDR) pathogens. Several factors were considered in the creation of this list, including mortality, healthcare load, community charge, the prevalence of resistance, the 10-year
tendency of resistance, transmission, prevention in the community, prevention in healthcare institutions, the ability to treat, and the pipeline. This priority list included carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, as well as third-generation cephalosporin- and carbapenem-resistant Enterobacteriaceae [12]. In relation to Gram-positive bacteria, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* were also present [12]. Pathogens that cause community-acquired infections, such as clarithromycin-resistant *Helicobacter pylori* and fluoroquinolone-resistant *Campylobacter* spp., *Neisseria gonorrhoeae* and *Salmonella typhi*, were also included [12].

### 1.2. PREVENTION

In terms of prevention, the CDC affirms that avoiding the development of infections reduces the levels of antibiotics used and reduces the propagation of resistant cells. The CDC is trying to prevent infections caused by antibiotic-resistant bacteria in healthcare settings, the community and food. The CDC works to avoid antibiotic resistance in healthcare settings by supplying a method to identify resistance, prescribing models at diverse scales and giving recommendations to healthcare facilities and laboratories through infection-control guidelines. To prevent antibiotic resistance in the community, the CDC is trying to implement systems to follow infections and their changes in resistance, organise teams in regional areas and at a national level and manage the transmission of infections. To prevent antibiotic-resistant foodborne infections, the CDC collaborates with health departments, with the Food and Drug Administration (FDA) (which regulates antibiotics, foods, animal feed and other products) and also with the U.S. Department of Agriculture (which is responsible for the regulation of meat, poultry and egg products). However, prevention is only the first step, and the development of new diagnostic tools to detect specific mechanisms of bacterial AMR is also an important issue in the fight against AMR.

### 1.3. ESKAPE PATHOGENS

The ESKAPE pathogens (*E. faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp.) are the main causes of nosocomial infections around the world. The majority of these pathogens are MDR isolates, which is one of the biggest challenges in clinical practice [4]. The main reason is that they are responsible for a dramatic increase in morbidity and mortality in infected patients, hence their prompt detection is vital [13].
Understanding the resistance mechanisms in these bacteria is not only critical for the development of new antimicrobial agents or other anti-infective treatments [4] but also for the development of new diagnostic techniques.

On one hand, the ability to detect antibiotic-resistance genes and their low turnaround times has made molecular methods a reference for the diagnosis of multidrug resistance. These methods have a high clinical and epidemiological impact [13]. Multiplex real-time polymerase chain reaction (PCR) merits special attention because it is able to quickly identify multiple pathogens and various resistant genes in a sample [14-19].

Moreover, the study of the bacterial mechanisms of tolerance and persistence to stress conditions (including antimicrobial agents), which could occur before the development of resistance, might provide the key to the fight against AMR. Although antibiotic-treatment failure is typically attributed to resistance, it has long been realised that other mechanisms (i.e. tolerance and persistence) help bacteria to survive antibiotic exposure. Several authors examined the progression from tolerant to resistant populations, observing that, as always, tolerance goes before resistance. This infers that avoiding the evolution of tolerance may be a new strategy for decelerating the occurrence of resistance [9]. The molecular mechanisms involved in the development of tolerant or persistent bacterial cells are numerous and include RNA polymerase, sigma S (RpoS) and the general stress response, oxidant tolerance (i.e. reactive oxygen species (ROS), energy metabolism or efflux pumps, the bacterial DNA damage (SOS) response, the quorum sensing (QS) system or bacterial communication, the 5’,3’-bis-guanosine penta/tetraphosphate [(p)ppGpp] network and toxin–antitoxin modules [10,20].

The minimum duration for killing (MDK), which is a quantitative measure of tolerance that can be extracted from time-kill curves, was proposed by Brauner et al. as a means to distinguish between the various strategies of bacterial survival under antibiotic stress. In clinical practice, the MDK concept may be helpful for different objectives, such as adjusting effective treatments depending on the specific survival strategies employed by the etiological agent and their duration [21].

1.4. NEW ANTIINFECTIVE TREATMENTS

The increased prevalence of antibiotic-resistant bacteria is one of the principal global health problems, therefore the discovery and development of new molecules and antimicrobial treatments is a main objective for the World Health Organization (WHO) [22]. In 2016, to raise awareness of the need for new antibiotics, WHO member states requested a priority list of antibiotic-resistant bacteria to perform research and develop new and beneficial
antiinfective treatments. The alternative antiinfective treatments against MDR pathogens are classified into the following seven groups: a) new drugs, b) phage therapy (including derivatives), c) antivirulence therapy, d) lysins, e) antibodies, f) probiotics and g) immune stimulation (Figure 1).

**Figure 1.** New antiinfective treatments. Due to the lack of effective antimicrobial agents to combat MDR bacteria, other anti-infective treatments should be considered. Immunotherapy, phage therapy and its derivatives, vaccines, new drugs, antivirulence compounds and probiotics, as shown in this figure, are alternative treatments to fight against infections caused by MDR bacteria.

**a) New drugs**

Since 2000, three new classes of human-use antibiotics have been launched on the market, one of which is restricted to topical use. ‘Gap innovation’ has been used to explain the absence of new structural types in the antibacterial arsenal since 1962 [20].

Two recent reports, one by the Infectious Diseases Society of America (IDSA) [24] and the other by the ECDC [25], demonstrated that there are a few candidate drugs in the pipeline that offer benefits over existing drugs and that a few of these drugs will treat infections caused by the ESKAPE pathogens. The
goal of the IDSA is to lay the foundations of a sustainable and global antibacterial drug R&D enterprise with the short-term capacity to develop 10 new, safe and effective antibiotics by 2020. To achieve this objective, the IDSA has released a new teamwork called the ‘10 x 20’ initiative. Specifically, the IDSA will sustain the development of 10 new systemic antibacterial drugs by means of discovering new drug classes as well as exploring potential new compounds from existing antibiotic families [26].

b) Phage therapy (including derivatives)

Bacteriophages (viruses that specifically infect bacteria) were discovered and used as antimicrobial agents during the 1920s; however, they stopped being used following the appearance of antibiotics. Nevertheless, they have continued to be used in the Soviet Union for decades. This ‘forgotten cure’ employs natural viruses that infect bacteria, and are present in all ecosystems, but are unable to infect eukaryotic cells [27].

The literature has described the use of living phages as a treatment for lethal infectious diseases caused by Gram-positive and Gram-negative bacteria. Another finding in the field of bacteriophage therapy is the possibility of treating with genetically-modified and nonreplicating phages. Moreover, bacteriophages are potential adjuvants of antibiotic therapy. Phages encoded with lysosomal enzymes are also efficient at treating infectious diseases [28].

Several animal studies have demonstrated the efficacy and safety of phage therapy in the treatment of different infections [29-31]. In humans, potential applications of phages include the phage-mediated prevention and phage treatment expanding from conventional phage therapy, treatment with phage enzymes (e.g. endolysins) and the use of phages as adjuvants of antibiotics. Lysins represent a new class of anti-infective agents that are obtained from bacteriophages. They are bacterial hydrolytic enzymes of the cell wall that are capable of selectively and rapidly [≥3 log colony-forming units (CFU) in 30 min] killing specific Gram-positive bacteria. They also provide a targeted therapeutic proposal that produces a limited effect in other bacteria. The potential for lysin resistance in bacteria should be low due to the direction of the highly-conserved peptidoglycan components [32]. Endolysins against Gram-negative pathogens were recently characterised and developed [33-35]. In S. aureus, the application of lytic proteins to treat severe infections such as bacteraemia or endocarditis has already been studied. Furthermore, the structure and mechanism of action of these proteins have also been examined to better understand their ability to inhibit the infection and to modify them to improve their activity [36].

Interestingly, the use of P. aeruginosa and A. baumannii phages together could inhibit QS systems [37,38]. These data highlight a new field in phage therapy.
In conclusion, phage therapy is a safe alternative for the treatment of infections caused by MDR pathogens. It can also be used in combination with existing antibiotics to enhance their effect; however, there are currently no approved phage applications for humans, and further clinical trials are needed in this area in the near future [27, 39].

c) Antivirulence therapy

The objectives of the antivirulence procedures are to reduce the use of antibiotics, reduce the appearance of antibiotic resistance and protect the beneficial flora. Antivirulence agents do not exert strong selective pressures on bacteria that benefit the evolution of resistance and persistence mechanisms and, because they do not have an impact on viability, they should not alter the beneficial microbiota [40]. Several techniques can be used to identify possible antivirulence compounds, including the scraping of natural products, the structural modification of native ligands and the in silico coupling and high-throughput screening (HTS) of chemical libraries. Research in this field has increased dramatically in recent years; however, the first antivirulence compound has yet to arrive.

Antivirulence strategies for ESKAPE pathogens tend to target: (a) specific virulence factors (e.g. type three secretion system (T3SS) and enterotoxins), (b) master virulence regulators and signals (e.g. two-component systems and QS, such as acetylases and lactonases) [41] or (c) resistance to host defences and antibiotics (e.g. capsules, staphyloxanthin and biofilms).

Vila-Farrés et al. proposed an innovative approach to tackling MDR bacteria. The outer membrane protein A (OmpA) is a beta-barrel porin that is highly conserved among bacterial species, especially Gram-negative bacteria. These authors studied the efficacy of OmpA inhibitors in the prevention of infection both in vitro and in vivo [42].

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Table 1. (continued)
d) Antibodies

In 1890, Von Behring and Kitasato were the first to use the blood of rabbits to neutralise toxins [94]. Since then, serum therapy has been widely used to treat infectious diseases such as pneumococcal pneumonia, meningococcal meningitis, dysentery and erysipelas. However, serum therapy has since been relegated to being used to treat scarce pathologies (i.e. hepatitis, measles or toxin-induced diseases) due to the high number of adverse effects observed. Following serum therapy, and as a result of advances in the field of immunology, the use of antibodies against a specific pathogen or virulence factor was implemented. Antibodies constitute a traditional boarding in infectious diseases that without question is not directly connected with resistance; however, the identification of determinants (which are involved in virulence) is relatively conserved among strains and could potentially be attractive to study in relation to resistance to multiple antibiotics [27].

e) Probiotics

Probiotics are live microorganisms that provide benefits for the health of the host when they are administered in suitable amounts [95]. The most commonly used probiotics are usually bacterial strains (i.e. Lactobacillus or Bifidobacterium) or fungal isolates of the normal microbiota (i.e. Saccharomyces boulardii). Probiotics interact through many paths, such as antimicrobial activity with growth inhibition or the expression of bacterial virulence agents. Probiotics generate acids that lower the pH of the local environment [96] and toxins that suppress the growth of other bacteria [27].

f) Immune stimulation

Attempting research and development to find the next generation of antibacterial drugs is essential; however, vaccines, in combination with the proper use of current antibiotics, are starting to be recognised as pivotal and potent tools to attenuate AMR [97]. Bacterial infections can be avoided with the preventive use of bacterial vaccines. As a consequence, antibiotic prescriptions will be reduced and the selective pressure of the drug that gives rise to resistant strains will be minimised. In addition, the beneficial effects of vaccines on AMR have also been perceived with viral vaccines, such as those that prevent influenza [98]. Such vaccinations can reduce inappropriate antibiotic prescriptions for a viral disease and avoid the bacterial superinfections that would require antibacterial therapy.

Newly developed vaccines (i.e. vaccines against infections caused by Clostridium difficile or S. aureus), pneumococcal-conjugate vaccines with wide serotype coverage and vaccines to avoid infections due to Gram-negative
bacteria promise to manage these severe diseases, encourage the reduced use of antibiotics and avoid AMR [99].

A new vaccine candidate (D-alanine auxotroph) against staphylococcal disease was recently developed [100]. Moreover, these authors also developed A. baumannii, P. aeruginosa and S. aureus mutants that were D-glutamate auxotrophic strains and proved their efficacy as whole-cell vaccines in vivo [101].

1.5. CONCLUSION

Many factors may be necessary to overcome MDR bacteria (‘superbugs’) including focusing on their prevention, the detection and development of new treatments where clinical involvement is essential, innovation and research. Collective global action is needed to manage the crisis of antibiotic resistance through the balancing of innovation access and stewardship.

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COMPETING INTERESTS

The authors declare that they have no competing interests.
Chapter 1

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Fighting antimicrobial resistance in ESKAPE pathogens


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Chapter 2

THE IMPRESSIVE ADAPTABILITY OF Acinetobacter baumannii: A PARADIGM OF ANTIMICROBIAL RESISTANCE

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Chapter 2

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2.1. INTRODUCTION

Nosocomial, or hospital acquired, bacterial pathogens frequently obtain and easily develop mechanisms of resistance to multiple antibiotics, presenting significant clinical and economic challenges. Globally, drug-resistant infections are currently responsible for more than half a million deaths each year. By 2050—unless new solutions are forthcoming—antimicrobial resistance will have caused the deaths of more than ten million people. Related healthcare costs worldwide are projected to be between 300,000 million and more than 1 billion dollars per year [1].

Recently, the World Health Organization (WHO) published a list of "priority" bacterial pathogens demonstrating antibiotic resistance [2]. Among these, *Acinetobacter baumannii* falls into the "maximum priority" category, and is considered one of the opportunistic pathogens most threatening to global health. *A. baumannii*'s high genetic plasticity allows it to adapt quickly to unfavourable contexts, and easily develop antibiotic resistance (which has led to multiple successive changes in therapeutic strategy). This pathogen has been cited by some authors as a paradigm of multidrug-resistance [3,4].

The increasing frequency of *A. baumannii* isolates that are resistant to antibiotics, such as cephalosporins, imipenem, sulbactam, rifampin, colistin or tigecycline, represents a major challenge when selecting appropriate treatment regimens. In recent years, the frequency of infections and hospital outbreaks caused by strains resistant to most available antibiotics has increased. Virtually all strains of *A. baumannii* are resistant to at least two antibiotic classes [5]. The carbapenems have classically been considered a last resort for the treatment of multidrug-resistant strains, but *A. baumannii* resistance to even this class of antibiotics has increased enormously during the first decade of the 21st century. The SENTRY antimicrobial surveillance program revealed a worldwide increase in imipenem resistance from 34–60% in only 3 years (the period from 2006–2009) [6]. In Spain, imipenem resistance rates were even higher, at 83%. Possibly the only effective antimicrobial against this pathogen (resistance rates <10%) is colistin [7], which has clinically-significant shortcomings, such as the high potential for nephrotoxicity and poor bioavailability in the lungs and cerebrospinal fluid [8,9]. Antibiotic misuse and insufficient investment in new drug development have resulted in few novel alternatives for the treatment of multidrug-resistant organisms reaching the market over the last two decades. The design and evaluation of new antibiotic therapies is imperative.

While *A. baumannii* lacks the necessary virulence factors to cause disease in healthy individuals, it can act as an opportunistic pathogen in susceptible individuals (*e.g.* those with underlying disease). Opportunistic pathogens can produce infection, which can only be prevented by the use of antimicrobial
therapies. *A. baumannii* can colonize niches where few other species would survive (e.g. environments with high antibiotic pressure) and can even displace commensal microflora. Antimicrobial resistance can increase the virulence or fitness of certain species in some environments, often helping these species to colonize new niches, such as the hospital environment. This explains why these pathogens are isolated much more frequently than few decades ago. Thus the genetic background of resistant pathogens such as *A. baumannii* allows it to persist in the presence of minimal concentrations of antibiotics or even in their absence [10]. The capacity to develop or acquire resistance and the ability to persist in complicated environments are key factors in explaining the increase in the number of infections in hospitalized patients [10]. Furthermore, in adapting to survival in the presence of antibiotics, species such as *A. baumannii* and *Pseudomonas aeruginosa* have evolved to cause greater host damage [11]. Carrying virulence genes also confers some evolutionary advantage during host colonization and infection, favouring resistant strains and providing a plasticity that allows *A. baumannii* to employ novel strategies in exploring new environments (including providing advantages over commensal microflora).

This review explores intrinsic and acquired resistance mechanisms of *A. baumannii*, with a focus on the main resistance transmission methods known thus far, in order to better understand the plasticity of this species in adapting to survival in the presence of antimicrobials and in a nosocomial environment.

### 2.2. MECHANISMS OF *A. baumannii* ANTIMICROBIAL RESISTANCE

The most relevant resistance mechanism in *Acinetobacter* spp. is enzymatic hydrolysis of β-lactams. However, due to complex resistance development and acquisition systems, multiple resistance mechanisms can coexist in this pathogen [12-14]. The main resistance mechanisms of *A. baumannii* are summarized in Table 1.
Table 1. Major resistance mechanisms of *A. baumannii* [3,15,16]

<table>
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<tr>
<th>Antibiotic</th>
<th>Resistance mechanism</th>
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<tr>
<td>β-lactams</td>
<td>Enzymatic inactivation</td>
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<td>Extended spectrum β-lactamases (AmpC, TEM, VEB, PER, CTX-M, SHV)</td>
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<tr>
<td></td>
<td></td>
<td>Carbapenemases (OXA, VIM, IMP, NDM-1)</td>
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<td></td>
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<td>Loss, down-regulation, or alteration of porins</td>
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<td>CarO, Omp 33-36, OmpD-like</td>
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<td>Alteration of PBP expression</td>
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<td></td>
<td>Efflux systems</td>
<td>AdeABC</td>
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<td>Aminoglycosides</td>
<td>AMEs</td>
<td>AAC, ANT, APH</td>
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<td></td>
<td>Efflux systems</td>
<td>AdeABC, AdeM</td>
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<td></td>
<td>Ribosomal methylation</td>
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<td>Tetracyclines and</td>
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<td>Polymyxin E</td>
<td>Lipid A modification</td>
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<tr>
<td>(Colistin)</td>
<td>Loss of lipopolysaccharide</td>
<td>LpxABC</td>
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### 2.2.1. Resistance to β-lactams

Inactivation of the β-lactams is due to the action of the β-lactamases enzymes, which are grouped into four molecular classes (A–D) following the Ambler’s Molecular classification system [17].

#### 2.2.1.1. Class A β-lactamases

*A. baumannii* exhibits a wide range of class A β-lactamases, including Temoneira (TEM), sulphydryl variable (SHV), cefotaxime-hydrolyzing β-lactamase (CTX-M), guiana extended-spectrum (GES), self-transferable plasmid from *Escherichia coli* (SCO), *Pseudomonas* extended resistant (PER), vietnam extended-spectrum β-lactamase (VEB), carbencillin hydrolyzing β-lactamase (CARB), and *Klebsiella pneumoniae* carbapenemase (KPC) [18]. However they use to have a minor role in resistance, especially in resistance to carbapenem antimicrobials. Class A β-lactamases, which are inhibited by clavulanic acid, typically hydrolyse penicillins and cephalosporins more effectively than carbapenems, with the exception of some KPC type enzymes [17].

Some members of this class – such as TEM-1, CARB-4, and SCO-1 – are narrow-spectrum β-lactamases, whereas other enzymes (*e.g.*, PER-1, TEM-92, CARB-10, SHV-5, PER-2, CTX-M-2, CTX-M-15, VEB-1, GES-14, and PER-7) are known
as extended spectrum β-lactamases (ESBLs) [8,19]. Clinically-relevant ESBLs include CTX-M enzymes, which (due to their transmission via plasmids or integrons) have a high potential for rapid dissemination and could thus produce drug-resistant infection outbreaks [20,21]. Although prevalent among Enterobacteriaceae, ESBLs are less prevalent in Acinetobacter spp., but CTX-M-2, CTX-M-43, and CTX-M-15 have been described from Japan, Bolivia and India, respectively [8]. Another relevant ESBL is VEB-1, with reported dissemination in France [22], Belgium and Argentina [8]. Finally, the PER ESBLs, initially observed in P. aeruginosa, have been detected in A. baumannii in many countries: in Korea, PER-1 is one of the most predominant β-lactamas [23,24]. Additional class A carbapenemases, such as GES-14 and KPC-2, have also been detected in A. baumannii [25,26].

A. baumannii also exhibits other class A β-lactamases such as the narrow-spectrum β-lactamases TEM-1 and -2, but they are of lesser clinical significance compared to other resistance mechanisms of Acinetobacter spp. [27,28].

2.2.1.2. Class B β-lactamases

This class comprises metallo-β-lactamases (MBLs), which require zinc or another heavy metals for antimicrobial catalysis [17], and are capable of hydrolysing all β-lactams except monobactams [29]. They exhibit broad-range, potent carbapenemase activity, and are resistant to inactivation by classical inhibitors [30].

MBLs are not the predominant carbapenemases in A. baumannii, but they do contribute to the high carbapenem resistance levels observed in this species. The firsts MBLs isolated in Gram-negative bacilli were imipenemase (IMP)- and Verona imipenemase (VIM)-type, in Japan and Italy, respectively [8]. IMP-1 and VIM-2 were the first of this class detected in Acinetobacter spp., in 1998 [31]. Subsequently, VIM-1, VIM-3, VIM-4, VIM-11, IMP-2, IMP-4, IMP-5, IMP-6, IMP-8, IMP-11, IMP-19 and IMP-24 have all been observed in this pathogen [18]. Seoul imipenemase (SIM)- and New Delhi metallo-β-lactamase (NDM) have also been detected in Acinetobacter spp. [8], although of the SIM-type MBLs, only SIM-1 has been detected [32]. Most recently, NDM-1 [33,34] and NDM-2 [35] have been detected in A. baumannii. The blaNDM−1 gene use to be integrated into the chromosome as a transposon, bracketed by two copies of ISAba125, which has facilitated its rapid dissemination [33,36,37].

The MBLs are especially predominant in non-baumannii Acinetobacter spp. Other genomic species (e.g. genomic sp. 13TU and genomic sp. 3) represent the majority of MBL-carrying Acinetobacter spp. isolates [38,39].

2.2.1.3. Class C β-lactamases

Most Gram-negative bacilli harbour a chromosomally-encoded β-lactamase, and this is usually a cephalosporinase belonging to class C [40]. Class C β-lactamases – like classes A and D – are serine-dependent, and display many
additional specific features, for example an insensitivity to clavulanic acid, and a preference for cephalosporin hydrolysis (although they can also confer resistance to cefamycins and penicillins) [29]. This confers significant therapeutic problems [19]. While the first AmpC β-lactamase characterized in *A. baumannii* was from a Spanish clinical isolate in 2000 [41], all strains actually possess intrinsic AmpC cephalosporinases [42] bearing small sequence differences [43,44]. In 2005, all *Acinetobacter* spp. chromosomal cephalosporinases were uniformly designated as *Acinetobacter*-derived cephalosporinase (ADC) β-lactamases [45]. ADC enzymes play a pivotal role in the antimicrobial resistance of *Acinetobacter* spp., especially in isolates carrying an insertion sequence under the control of a strong promoter (ISAba1-like sequence), which usually results in pronounced resistance to ceftazidime [47,48].

2.2.1.4. Class D β-lactamases

Also known as the OXA-type β-lactamases (in reference to their preferred hydrolysis of oxacillin) [29], this is probably the fastest-growing class of β-lactamases with more than 500 reported enzymes [49]. The class D enzymes are organised into three categories, based on substrate specificity: narrow-spectrum (*e.g.* OXA-1 and -10), extended-spectrum β-lactamases (ESBLs, *e.g.* OXA-13 and -17), and carbapenem-hydrolysing class D β-lactamases (CHDLs, *e.g.* OXA-23 and -24/40) [49,50]. Approximately 50% of OXA β-lactamases possess carbapenemase activity [51]. However, due to the multiple mechanisms of antibiotic resistance exhibited by *A. baumannii*, it is difficult to conclusively determine the relative contribution of CHDLs [52].

Until a decade ago, the most clinically relevant β-lactamases were the AmpC and extended-spectrum β-lactamases, but the number of known carbapenemase β-lactamases has increased in recent years, with CHDLs being especially problematic. Since the description of the first OXA enzyme (OXA-23) in *A. baumannii* in 1993, the number of CHDLs discovered worldwide in clinically-problematic Gram-negative pathogens, such as the *Enterobacteriaceae*, *P. aeruginosa*, and (largely) *A. baumannii* has increased dramatically [53,54]. Among the four classes of β-lactamases, MBLs and CHDLs are the main groups of carbapenemases occurring in *A. baumannii*, and degradation is the most common mechanism of carbapenem resistance [55,56].

*A. baumannii* possesses six CHDL families: OXA-23-like, OXA24/40-like, OXA-58-like, OXA-143-like, OXA-235-like and OXA-51-like [57-60]. Of these, OXA-23-like is the major family of CHDLs, the most disseminated worldwide, and the main source of carbapenem resistance in this pathogen [61]. Other prevalent groups include OXA24/40-like and -58-like. Nearly all strains of *A. baumannii* possess OXA-51-like chromosomal enzymes with weak carbapenemase activity. However, if the gene acquires a strong promoter via
upstream insertion sequence ISAbA1, or is located in a plasmid, it can confer carbapenem resistance [59,62]. The aforementioned insertion sequence has also been described to increase expression of OXA-23 and -58 [63].

The OXA-24/40 CHDL group comprises OXA-24/40, OXA-25, OXA-26, and OXA-72 [58]. OXA24/40 was initially identified in Spain in 2000 [64]. Later, OXA-25, OXA-26 and OXA-27 were identified in A. baumannii clinical isolates originating in Spain, Belgium and Singapore, respectively [65]. This group of carbapenemases has now been identified worldwide [18].

OXA-58 was first identified in France, encoded within a plasmid from a multidrug-resistant A. baumannii isolate, in 2005 [66]. Since then, it has been found that OXA-58-like CHDL-producing A. baumannii isolates are widely distributed [168].

Given that carbapenems have been utilized as last-resort antibiotics for the treatment of multidrug-resistant Acinetobacter infections, the prevalence and plasmid-mediated dissemination of CHDLs are an important clinical challenge, motivating evaluation of potential alternate antibiotics (including β-lactamase inhibitors, aminoglycosides, tigecycline and polymyxins) in the treatment of such infections.

2.2.1.6. Outer membrane proteins

Although β-lactamases with carbapenemase activity are the main carbapenem resistance mechanism, porins are also thought to be involved. Their reduced expression plays a role in the resistance to these antibiotics. Few outer membrane proteins (OMPs) have been reported, their functions remain unclear [67], and – compared to other pathogens – little is known about the porins of A. baumannii. Indeed, A. baumannii exhibits very low outer membrane permeability, with a small number and size of porins relative to other Gram-negative organisms [68]. Several reports describe down-regulated expression of some OMPs, implicating these in antimicrobial resistance [13,14]. The major OMP of A. baumannii, reported to date, is the heat-modifiable protein HMP-AB, which exhibits homology with the monomeric OmpA of Enterobacteriaceae and with OMP-F (OprF) of P. aeruginosa [69]. Three additional porins implicated in carbapenem resistance are the 33–36 kDa protein [70], the 29 kDa protein (CarO: carbapenem resistance-associated outer membrane protein of A. baumannii) [71] and the 43 kDa protein, which exhibits homology with OprD of P. aeruginosa [72]. It has been suggested that CarO may function as a carbapenem-nonspecific channel, while OprD-like porin may function as a carbapenem-specific channel [73]. Clinical outbreaks of carbapenem-resistant A. baumannii isolates have been described in association with porin loss, including loss other OMPs, such as 47-, 44-, and 37-kDa OMPs in A. baumannii isolates in New York City [12], and loss of 22- and 33-kDa OMPs in association with OXA-24/40 in Spain [14].
2.2.1.7. Multidrug efflux pumps

While the outer membrane can limit the entry of antimicrobials into the bacterial cell, multidrug efflux pumps actively export multiple classes of antimicrobials from the cell [73]. While transport proteins involved in metabolic function exhibit a high degree of substrate specificity, multidrug efflux systems are more promiscuous [74]. Thus, efflux pumps work synergistically with the low permeability of the outer membrane [75]. Expression of efflux pumps is associated with an increased minimum inhibitory concentration (MIC) of many different antibiotic classes, including resistance to tigecycline, aminoglycosides and carbapenem in *A. baumannii* [76].

The major efflux pumps involved in multidrug resistance in *A. baumannii* belong to the group of proton-motive-force-dependent exporters, especially the major facilitator superfamily (MFS) and resistance-nodulation-cell division superfamily (RND) families [73]. Several MFS-family efflux pumps have been characterized in *A. baumannii*, including TetA, CmlA, MdfA, CraA and AmvA, which mediate resistance to different types of antibiotics (including β-lactams) [18]. An example of an RND family member is (chromosomally-encoded) AdeABC of *A. baumannii*, the best studied member, thus far. AdeABC overexpression is a major mechanism for decreased susceptibility to various antibiotic classes [76], and efflux inhibitors (*e.g.* phenyl-arginine-β-naphthylamide, carbonyl cyanide 3-chlorophenylhydrazone) have been shown to reverse resistance [77].

Since antibiotics act as AdeABC substrates, they can increase the expression of the AdeABC genes, which are chromosomally encoded, leading to multidrug resistance. Treatment failure and death due to *A. baumannii* infection are common when efflux pumps (especially the RND family) are implicated in antibiotic resistance [78]. In addition to the AdeABC efflux pump, other RND-type efflux pumps, including AdeFGH [79] and AdeIJK [80], are implicated in multidrug resistance in *A. baumannii*.

2.2.1.8. Penicillin-binding proteins

Another relevant mechanism of resistance to β-lactams is direct modification of their targets: the penicillin-binding proteins (PBPs), which catalyse peptidoglycan synthesis and are associated with cell morphogenesis and the cell division complex [81]. β-Lactams are suicide inhibitors which bind covalently and irreversibly to PBPs [82]. There is limited information regarding this resistance mechanism in *A. baumannii*, but no differences were found between the sequences of susceptible and resistant *A. baumannii* strains [83]. Although confirmatory studies are required, this suggests that PBP mutations are not as important a resistance mechanism in *A. baumannii* as in other pathogens (*e.g.* *P. Aeruginosa*) [84].
However the impact of PBP alterations on bacterial virulence should be further investigated, as PBP7/8 contributes to both the \textit{in vitro} and \textit{in vivo} survival of \textit{A. baumannii}. A PBP7/8 mutant strain exhibited poorer survival in \textit{in vivo} models, compared to the isogenic wild-type strain [85].

2.2.1.9. Resistance to β-lactamase inhibitors

Most β-lactamase inhibitors are ineffective against \textit{A. baumannii}: no commercial inhibitors exist with activity against its class B and D carbapenemases. An exception is sulbactam, which has affinity for \textit{A. baumannii}'s PBPs [13,86] and possesses bactericidal activity against \textit{A. baumannii}. It is usually used in combination with ampicillin; however, the contribution of sulbactam is more relevant than the contribution of ampicillin as antimicrobial agent against this pathogen. While no breakpoints have been defined for \textit{A. baumannii}, it is considered susceptible when MIC ≤ 4 mg L$^{-1}$ [87]. Sulbactam monotherapy is not recommended in patients with serious infections, and resistance to sulbactam has already been described in \textit{Acinetobacter} [87]. Reduced PBP2 expression is associated with resistance to this compound [13], although production of the non-ESBL β-lactamase TEM-1 has also been suggested to contribute to sulbactam resistance [88]. Clavulanic acid, too, shows bactericidal activity against a percentage of \textit{A. baumannii} isolates [89]. New agents, which specifically inhibit \textit{A baumannii} β-lactamases, are being developed (\textit{e.g.} ETX2514 [90] and LN-1-255 [60]), but additional preclinical assays are required to evaluate their \textit{in vivo} efficacy.

2.2.2. Resistance to aminoglycosides

Aminoglycosides are effective against both Gram-negative and positive pathogens. During \textit{A. baumannii} infection, specifically, tobramycin and amikacin are used (in combination with other antimicrobials) [9]. Aminoglycoside antibiotics target ribosomal 16S rRNA, modifying its structure to produce a loss of translation fidelity, production of erroneous proteins, and, finally, bacterial cell death [91]. Bacteria exhibit increasing levels of resistance to aminoglycosides, mainly by acquiring genes encoding aminoglycoside-modifying enzymes (AME), \textit{N}-acetyltransferases (AAC), phosphotransferases (APH) or \textit{O}-adenyltransferases (ANT), all of which are typically encoded by transposable elements. Other implicated mechanisms of resistance include mutation of ribosomal proteins or RNA. Ribosomal methylases – which methylate the aminoacyl site of 16S rRNA – also confer high resistance to aminoglycosides, and six acquired 16S rRNA methyltransferases have been described: \textit{armA}, \textit{rmtA}, \textit{rmtB}, \textit{rmtC}, \textit{rmtD} and \textit{rmtE} [92]. An additional mechanism of aminoglycoside resistance is reduction of intracellular accumulation by means of outer membrane alterations, two-component systems and efflux pumps [\textit{e.g.} the AbeM pump, a member of the multidrug and toxic compound extrusion (MATE) family] [93].
Many *A. baumannii* isolates express a combination of aminoglycoside-modifying enzymes [94,95]. A multidrug resistant *A. baumannii* isolate carrying genes for four aminoglycoside-modifying enzymes was described in China [96], and Japanese and Greek studies have shown that the majority of multidrug-resistant isolates carry at least one gene encoding an aminoglycoside-modifying enzyme [95,97]. In a 2007 outbreak of highly aminoglycoside-resistant *A. baumannii* isolates, the strains carried the genes for *armA* together with Per-1 and OXA-23 carbapenemases [23]. Similarly, multidrug-resistant *A. baumannii* isolates have been described that carry both a *bla*OXA-23-like gene (*aac(6’)-Ib*) and the 16S rRNA methylase *armA*. Such isolates show that *A. baumannii* can employ multiple simultaneous mechanisms to elude the action of antimicrobials.

### 2.2.3. Resistance to tigecycline

Tigecycline is a relatively new broad-spectrum glycylcycline antibiotic, which has demonstrated *in vitro* and *in vivo* activity against *A. baumannii* [98]. It is a derivative of minocycline, with structural modifications that improve ribosomal binding-site affinity and orientation relative to both minocycline and tetracycline [99]. Tigecycline is one of the few agents developed recently that has enhanced activity against problematic Gram-negative organisms. In the 2005–2011 Tigecycline Evaluation and Surveillance Trial investigating multidrug-resistant *A. baumannii* strains, tigecycline’s MIC$_{50}$ was 0.5 mg L$^{-1}$, and its MIC$_{90}$ was 1 mg L$^{-1}$ [100].

Tigecycline can often overcome active efflux and ribosomal protein resistance mechanisms which inactivate the tetracyclines (*tet*(A) to *tet*(E) efflux pump genes) [101]. However, resistance has been detected in some strains, due to high expression levels of chromosomally-encoded efflux pumps; tigecycline is susceptible to efflux by overexpressed multidrug efflux systems such as AdeABC and AdeIJK [101,102]. Several studies have already described high rates of tigecycline-resistant strains in *A. baumannii* [103,104], including development of resistance during tigecycline treatment [105]. However, little is known about the specific tigecycline resistance mechanisms of *A. baumannii*, necessitating further investigation.

### 2.2.4. Resistance to polymyxins

The increasingly frequent isolation of strains resistant to carbapenems, sulbactam, rifampin or tigecycline [106,107] has driven the therapeutically use of polymyxins. Colistin and polymyxin B bind to the portion of the cell membrane interacting with the lipid A moiety of lipopolysaccharide (LPS) to cause outer membrane disorganization and hyper-permeability, leading to rapid Gram-negative bacterial death [108]. Colistin – discovered in the 1940s – was used mainly in the 1960s and 1970s, before being largely abandoned due to
nephron- and neurotoxicity; recent studies have demonstrated that revised dosing regimens can minimize this problem [109].

Polymyxins are active against most *A. baumannii* isolates, though colistin-resistant isolates have been described *in vitro* and *in vivo* [110-114]. For example, of the *A. baumannii* isolates recovered at two Korean hospitals, 30.6% exhibited colistin resistance [115], and outbreaks of polymyxin-resistant *A. baumannii* have been already reported [116-118]. Two primary colistin-resistance mechanisms have been described in *A. baumannii* to date. The most common is modification of the lipid A moiety of LPS with phosphoethanolamine (PEtN) as a result of mutations in the *pmrA/pmrb* two-component system [119]. The modifications of lipid A by the addition of PETN confer a positive charge to LPS, preventing colistin binding. The mutations in *pmrA* or *pmrB* induce expression of *pmrA*, in turn leading to up-regulated expression of the *pmrCAB* operon, and subsequent synthesis and addition of PETN to the lipid A portion of LPS [119-121]. The second mechanism is the complete loss of lipopolysaccharide caused by either mutation or insertional inactivation of the lipid A biosynthesis genes. In colistin-resistant *A. baumannii*, mutations in lipid A biosynthesis genes (*lpxA*, *lpxC* and *lpxD*) due to nucleotide substitution, deletion or insertional inactivation by sequence IS*Aba11*, completely abrogate production of LPS with high colistin resistance (MIC > 128 mg L\(^{-1}\)) [113,122]. Additionally, polymyxin B-resistant *A. baumannii* isolates have been shown to carry mutations in the *lpxC* and *lpxD* genes, in addition to mutations in the *lpsB* gene which encodes a glycosyltransferase involved in LPS core biosynthesis [123]. However, colistin-resistant *A. baumannii lpx* mutants (lacking LPS) also demonstrate reduced virulence and fitness compared to colistin-resistant *A. baumannii pmrA/pmrb* mutants (PETN-modified LPSs) [124], indicating that *lpx* mutation comes with a biological cost.

Other colistin-resistance mechanisms have been recently suggested, including modification of Gram-negative outer membrane asymmetric lipid distribution (essential for outer membrane functions) [125]. Also, two studies have suggested that efflux pumps may be involved in the colistin resistance phenotype in *A. baumannii* [126,127].

**2.3. VIRULENT AND MULTIDRUG-RESISTANT DISSEMINATED CLONES OF *A. baumannii***

Multidrug-resistant microorganisms, such as *A. baumannii*, are opportunistic pathogens, able to compete in new niches where previously only commensals or non-pathogenic microorganisms existed. The ability to adapt to survival and persistence in nosocomial environments, encompassing patients with
The impressive adaptability of \textit{Acinetobacter baumannii}...

weakened immune systems and an environment with high antimicrobial pressure, has led to the emergence of \textit{A. baumannii} as a key pathogen, whereas a few decades ago, it caused practically no disease. At least in these specific settings, the incidence of multidrug-resistant and virulent clones of \textit{A. baumannii} is also increasing worldwide.

The population of clinical isolates of \textit{A. baumannii} is dominated by three lineages: European or International clones I, II and III, corresponding to clonal complex 1 (CC1, comprising ST1, ST7, ST8, ST19 and ST20), clonal complex 2 (CC2, comprising ST2, ST45 and ST47) and clonal complex 3 (CC3, comprising ST3 and ST14) \cite{128,129}. The predominance of a few successful multidrug-resistant lineages worldwide underlines the importance of studying \textit{A. baumannii} epidemiology.

Outbreaks are most frequently attributable to European clones I and II \cite{130}. The evolutionary advantage of these predominant clones is due to their capacity for acquiring resistance determinants. Clone II is particularly well-adapted to hospital environments \cite{131}, and CC2 is the most frequent genetic lineage observed in European, Asian and North American carbapenem-resistant \textit{Acinetobacter} isolates (with carbapenem resistance mainly mediated by class D \(\beta\)-lactamases such as OXA-23, OXA-24/40 and OXA-58, or relevant metallo-\(\beta\)-lactamase-type carbapenemase genes) \cite{132-134}. Other CCs that exhibit narrower distribution patterns than European clones I–III do exist, such as clone ST15. The predominance and geographic distribution of certain clones is usually associated with their capacity to acquire multiple resistance mechanisms \cite{128}, either chromosomal or plasmid-encoded \cite{135}. Regarding geographic distribution of acquired OXA genes, OXA-58-like genes are associated with Greece and Italy, OXA-24/40-like genes are associated with Spain and Portugal, and OXA-23-like genes are associated with Northern European countries, Asia and South America \cite{129}. The ability of these clonal lineages to adapt and acquire these mechanisms is a key role in the successes of their distribution.

Frequently, internationally-distributed resistant clones demonstrate efficient pathogenic factors. For instance, an isolate carrying the OXA-23 carbapenemase gene, and belonging to international clone II, led to the death of a patient within only six days \cite{136}. Some clones can cause outbreaks, rapidly affecting up to hundreds of patients. For example, the high-risk clone ST56, which is susceptible only to tigecycline and colistin, and carries the carbapenemase OXA-24/40, as well as overexpressing two putative virulence factors (septicolyisin and the TonB-dependent receptor). This strain spread extremely rapidly, causing the largest nosocomial outbreak ever reported, with 377 patients becoming colonized or infected with \textit{A. baumannii} \cite{137}.

While \(\beta\)-lactamase-mediated resistance to \(\beta\)-lactams is of great therapeutic concern, few studies have analysed the impact of these enzymes on \textit{A. baumannii} virulence. There is, however, a relationship between carrying
carbapenemase PER-1 and increased cell adhesion in *A. baumannii* strains, although the exact mechanism of the association remains unknown [138]. Other resistance factors are also implicated in virulence. Porins, for example, provide trans-membrane passage for molecules, such as nutrients, toxins and antibiotics, and have clear roles in both virulence and resistance [139]. Furthermore, the *A. baumannii* OmpA (HMP-AB) protein, which has been associated with cephalosporin resistance [140], has also been reported to induce human epithelial and dendritic cell death via mitochondrial targeting [141], and is involved in biofilm formation [142]. The Omp33-36 protein of *A. baumannii*, involved in carbapenem resistance, has also been suggested to be involved in apoptosis and modulation of autophagy [143]. Moreover, both CarO and Omp33-36 porins are implicated in biofilm formation [144], and both OmpA and Omp33-36 proteins (as well as the TonB-dependent copper receptor) are fibronectin-binding proteins (FBPs) [145].

### 2.4. ANTIMICROBIAL RESISTANCE AND VIRULENCE MECHANISM CO-SELECTION

*A. baumannii* is naturally competent at incorporating DNA from other bacterial species [19]. It is commonly-known that the horizontal transfer of multiple resistance genes can occur simultaneously. Similarly, horizontal transfer is a relevant mechanism to exchange virulence factors. The co-selection of virulence and resistance factors has been observed during pathogen evolution in the post-antibiotic era, and could contribute to bacterial adaptation to new environments. The distribution of such transfer elements (*e.g.* plasmids, integrons and transposons) in pathogens, such as *Acinetobacter*, may become a major clinical challenge in the future.

Plasmids are extra-chromosomal, self-replicating elements, which are non-essential and are usually implicated in functions, such as virulence, resistance or persistence under extreme conditions [3]. Conjugative plasmids have a relevant role in the evolution of *A. baumannii* due to their horizontal transmission, including resistance genes (mainly during the last decades), highlighting the key role of the plasmids [146,147]. Evidence suggests that *A. baumannii* strains may bear distinct sets of plasmid types and thus a broad heterogeneity of resistance plasmids [148,149]. An example of virulence and resistance co-selection in *A. baumannii* has been described in clone ST56, which caused a large 2006 outbreak in a Spanish hospital. The plasmid pMMA2, isolated from the main outbreak clone, harboured the *bla*OXA-24/40 gene, as well as two genes implicated in virulence (*a*septicolysin-like gene encoding a pore-forming toxin, and a TonB-dependent receptor gene encoding an outer membrane protein involved in iron uptake) [137].
Genetic elements associated with antibiotic resistance gene acquisition by *Acinetobacter* include integrons and transposons. Integrons contain site-specific recombination systems through which they can include resistance genes, and transposons are able to integrate and move genes. These structures are usually found as part of the chromosome or plasmids [150-152]. There is a high prevalence of class 1 and 2 integrons in *A. baumannii* [153,154]. Most acquired MBL genes in *A. baumannii* occur within class 1 integrons, which often contain several resistance genes. Many genes encoding aminoglycoside-modifying enzymes are associated with transposons. Moreover, transposons can concomitantly carry other resistance determinants [24].

IS elements, too, play an important role in *A. baumannii* resistance. IS*Aba*1, IS*Aba*2, IS*Aba*3, IS*Aba*4 and IS18 are associated with carbapenemase gene expression in *A. baumannii*. As intimated above, IS*Aba*1 is the most highly-prevalent such element of this pathogen [155]. Frequently, IS element-encoded promoter regions increase expression of genes such as *bla*OXA-23-like, *bla*OXA-51-like, *bla*OXA-58-like and *bla*AmpC [151].

Gram-negative bacilli secrete vesicles, which mediate interactions with other bacteria and eukaryotic cells in the local environment. The *A. baumannii* secretome is also implicated in both virulence and resistance; two examples of outer membrane vesicles (OMVs) of the secretion systems transporting virulence and resistance factors have been described. OMV-mediated toxin delivery is a potent virulence mechanism employed by various Gram-negative organisms. The OMVs can thus be employed as a means to deliver virulence factors, and it appears they may also be relevant to transmission of resistance genes in *A. baumannii*. The release of OMVs favours the spread of antibiotic resistance genes to other bacteria *via* horizontal DNA transfer (*e.g.* transmission of OMVs harbouring the *bla*OXA-24/40 carbapenemase gene in an *A. baumannii* isolate). Furthermore, the *A. baumannii* ATCC 17978 strain, transformed with OMVs from clinical strains, exhibits an antibiotic-resistant profile due to expression of OXA-24/40 carbapenemase. Thus, in *A. baumannii*, OMVs represent a new mechanism of antibiotic resistance gene dissemination, in addition to the previously-known mechanisms (conjugation, transformation, and transduction) [156].

Lastly, some Gram-negative bacteria utilize the antibacterial type VI secretion system (T6SS) to kill competitors. In *A. baumannii* strains, T6SS exhibits variable expression, including a self-transmissible plasmid that carries T6SS negative regulators, as well as resistance factors. This secretion system is inactivated in resistant cells, but some cells lose this plasmid, thereby derepressing expression of the secretion system and T6SS-mediated killing of the rest of surrounding microflora. In this way, harbouring of resistance genes can lead to elimination of competing bacteria [157].
2.5. CONCLUDING REMARKS

This review highlights the major resistance mechanisms of *A. baumannii*, and their role in the spread of this organism. Future considerations include the urgent requirement for new antibiotics to treat emerging bacteria that are resistant to almost all existing (including last-resort) antibiotics. Very few truly new antibiotics have been developed against such Gram-negative pathogens that have emerged since the 1990s. Development of new antibacterial therapies need not be limited to antibiotics; innovative anti-virulence therapies (including novel vaccines and the revival of antibacterial phage therapy) are a promising alternative to antibiotic treatment in combating resistant and/or virulent pathogens.

ACKNOWLEDGMENTS

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The impressive adaptability of Acinetobacter baumannii...
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ANTIMICROBIAL RESISTANCE IN STAPHYLOCOCCI

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Chapter 3

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3.1. INTRODUCTION

Staphylococci are wide-spread commensal bacteria that colonise the skin and mucous membranes of humans and animals. The presence of numerous virulence factors makes these bacteria opportunistic pathogens [1], which, under suitable conditions, may become harmful to the host.

Pathogenicity was first only attributed to coagulase-positive staphylococci (CoPS). Recently, this opinion has changed because of the discovery of virulence genes among coagulase-negative staphylococci (CoNS). Moreover, CoNS are seen as potential donors of resistance genes for not only closely related bacterial genera. A particularly serious problem is the transfer of resistance genes between CoNS and human or animal pathogens. It has to be realised that CoNS could significantly increase the pathogenic potential of other bacteria and, via the transfer of resistance genes, may decrease the possibilities of disease control because of treatment failure.

The discovery of penicillin in 1928 by the Scottish scientist Sir Alexander Fleming has marked a new era in the treatment of staphylococcal infections in both human and veterinary medicine. The production and application of antibiotics have increased within a few of years to such an extent that the twentieth century could be referred to as „the century of antibiotics“.

However, the miracle of antibiotics soon collapsed with the emergence of the first resistant bacterial strains. Staphylococci have begun to develop effective mechanisms to eliminate the lethal power of antibiotics. Upon permanent contact with antibiotics, these bacteria activated their defence mechanisms and changed their metabolic pathways to win this fight against humankind. Staphylococci are able to persist under extreme environmental conditions for a long time, as evidenced by the recovery of two Staphylococcus succinus strains from 25–35-million-year-old Dominican amber [2]. Because of their extreme adaptability, it is practically impossible to eliminate staphylococci from the environment. Therefore, these bacteria have to be acknowledged, tolerated and understood to be able to predict their behaviour and to minimise the risk for humans and animals.

The phenomenon of antimicrobial resistance can affect any country of the world. Mechanisms of antimicrobial resistance can spread globally among microbial species and genera and are not fully understood. Nowadays, resistant strains of staphylococci can be found in the environment, in animals, humans and various food products. Therefore, the dissemination of resistance genes poses a challenge for both human and animal health professionals [3]. Across the globe, microbiologists and pharmacologists investigate the microbial mechanisms of drug inactivation and synthesise new generations of antibiotics in an attempt to control bacterial infections. However, it is more
than likely that staphylococci will soon develop other effective tools to fight the new drugs, thereby repeating the cycle.

In the last decades, the emergence of the methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE) has been a major concern. Patients with infections caused by resistant microbial strains are at higher risk of death than patients infected with non-resistant strains of the same bacteria. As reported by the World Health Organization, people infected with MRSA are estimated to be 64% more likely to die than people with a non-resistant form of the infection [4]. Hospital-acquired and health-care related infections caused by multidrug-resistant strains of staphylococci require longer hospital stays and higher costs for medical treatment, with increased mortality and decreased effects of the antimicrobial therapy. Permanent increase in antimicrobial resistance leads us to the conclusion that the twenty-first century will probably be referred to as „the century of antimicrobial resistance”.

Currently, the food chain is seen as one the most important pathways of spreading antimicrobial resistance. Sufficient heat treatment of processed food products will stop the activity of vegetative bacterial cells. However, pasteurisation temperatures destroy neither bacterial DNA nor mobile genetic elements encoding antimicrobial resistance. Although the vegetative cells are devitalised, the resistance genes remain in a particular food product. After consumption, these genes can be incorporated into the genome of other viable bacteria in the digestive tract of the host.

The occurrence of resistant staphylococci in drinking water and wastewaters may also represent a hazard, particularly under conditions capable of favouring their overgrowth [5]. Municipal wastewaters can also serve as reservoirs for MRSA dissemination [6]. If wastewater treatment methods will not be changed soon, resistance genes present in the effluents from hospitals and animal slaughterhouses will continue to persist in the environment. Resistant CoNS strains have also been found in free-living animals. These animals move throughout the environment without any boundaries and can therefore easily come into contact with agricultural and municipal wastes as well as food-producing animals and pets, making them potential reservoirs for resistance genes. The presence of genes encoding antimicrobial resistance is no always accompanied with their phenotypic expression, as some genes may remain dormant. Therefore, phenotypic susceptibility is a better criterion when considering treatment options [7].

The problem of antimicrobial resistance requires an international approach of all participants, including politicians, health professionals, farmers, health-care and food industry experts, as well as all members of the society. There is a general need develop new, effective fighting strategies based on prevention via national action plans developed by individual countries, surveillance of infections caused by resistant strains, effective control measures, investments
in the development of new medicines and diagnostics tools as well as permanent information of the public on the impact of antibiotic resistance. Antimicrobial resistance is a problem of global public health and food security and requires coordinated efforts from all countries.

### 3.2. CHARACTERISTICS OF THE GENUS *STAPHYLOCOCCUS*

Staphylococci are ubiquitous Gram-positive, spherical, nonmotile bacteria that can be found on the skin and mucous membranes of warm-blooded animals and humans, in environmental resources (*e.g.* air, soil, sand, dust or natural waters), as well as in various food products such as milk, meat or cheese [1]. Due to the typical structure of their cell wall, which comprises peptidoglycan and teichoic acid, staphylococci are highly tolerant to drying and dehydration [8].

Based on comparative 16S rRNA sequence analysis, the genus *Staphylococcus* belongs to the phylum *Firmicutes*, class *Bacilli*, order *Bacillales* and family *Staphylococcaceae* [1]. Currently, more than 50 species and almost 30 subspecies are recognised among staphylococci. The three most recent species, described in 2015, include *S. argensis* [9], *S. argenteus* and *S. schweitzeri* [10]. Most strains of staphylococci are catalase-positive and oxidase-negative, grow at temperatures between 18 and 40 °C and tolerate the presence of 10 % NaCl. In general, staphylococci are resistant to lysis by lysozyme, low levels of erythromycin and bacitracin, but susceptible to furazolidone, nitrofuran and lysis by lysostaphin [1].

According to plasma coagulation, staphylococci are classified as coagulase-positive staphylococci (CoPS) or coagulase-negative staphylococci (CoNS). Coagulase is an enzyme that reacts with prothrombin in the blood. The resulting *staphylothrombin* complex enables the enzyme protease to convert fibrinogen to fibrin, resulting in blood clot formation. Two types of coagulase are recognised in staphylococci, bound coagulase and free coagulase. While free coagulase is a heat-labile enzyme that is secreted extracellularly, bound coagulase is a cell wall-associated protein. Free coagulase binds with coagulase-reacting factor (CRF) in the blood and forms the complex *staphylothrombin*. Bound coagulase is also known as *clumping factor*. Upon contact with blood, the fibrin cloth protects the bacterium from phagocytosis and other host defence mechanisms [11]. Most human *S. aureus* isolates possess both forms of coagulase [12,13].

The formation of the coagulase enzyme is encoded by the *coa* gene, which is highly polymorphic [14]. Detailed gene analysis has revealed variable sequences in the 3'-end coding region of allelic gene forms [15], particularly in the C-terminal region containing the 81 bp tandem short sequence repeats.
(SSRs) encoding repeated 27 amino acid sequences [14,16]. Therefore, various sizes of coa gene have been observed and reported [17-19]. Because of a close correlation between coagulase formation and the pathogenicity of staphylococci, detecting the coagulase enzyme is still of great clinical importance [14,20].

<table>
<thead>
<tr>
<th>Species group</th>
<th>Species (subspecies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coagulase-positive and novobiocin-susceptible</td>
<td><em>Staphylococcus intermedius</em> S. delphini S. intermedius S. pseudointermedius S. schleiferi subsp. coagulans S. aureus</td>
</tr>
<tr>
<td>Coagulase-negative and novobiocin-susceptible</td>
<td><em>Staphylococcus epidermidis</em> S. capitis S. caprae S. epidermidis S. haemolyticus S. hominis S. saccharolyticus S. schleiferi subsp. schleiferi S. simiae S. warneri</td>
</tr>
<tr>
<td>Coagulase-negative and novobiocin-resistant</td>
<td><em>Staphylococcus simulans</em> (β-galactosidase-positive) S. carnosus S. felis S. simulans</td>
</tr>
<tr>
<td><em>Staphylococcus saprophyticus</em></td>
<td>S. arlettee S. cohnii S. equorum S. galinarum S. kloosii S. neapensis S. saprophyticus S. succinus S. xylosus</td>
</tr>
<tr>
<td>Coagulase-variable and novobiocin-resistant</td>
<td><em>Staphylococcus sciuri</em> (oxidase-positive) S. lentus S. sciuri S. vitulinus S. fleurettii</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> possess a long list of virulence factors. These include surface proteins necessary for colonisation and secreted proteins that allow invasion of and damage to host cells and tissues. Surface proteins include <em>clumping factor</em>, protein A, capsular polysaccharide adhesin as well as...</td>
<td></td>
</tr>
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</table>
fibronectin- and collagen-binding proteins. Secreted are numerous toxins (α-, β-, γ- and δ-toxins, exfoliate toxins, enterotoxins, toxic shock syndrome toxin, Panton-Valentine leukocidin) and various enzymes (catalase, deoxyribonuclease, hyaluronidase, β-lactamase, staphylokinase, lipases) [21,22].

Based on DNA-DNA hybridisation studies, staphylococci have been divided into several species groups [8]. Individual species (subspecies) with clinical significance are shown in Table 1.

### 3.3. CLINICAL SIGNIFICANCE OF STAPHYLOCOCCI

Virulence factors are mostly associated with coagulase-positive staphylococci. Among them, *S. aureus* is the most significant human pathogen responsible for a variety of respiratory, wound, skin and soft tissue infections, such as pneumonia, folliculitis, furuncles, carbuncles, impetigo, myocarditis, pericarditis, acute endocarditis, osteomyelitis, enterocolitis, mastitis, cystitis, prostatitis, cervicitis, meningitis or bacteraemia [1]. The main constituents of the cell wall of *S. aureus* are peptidoglycan, teichoic acid, fibronectin-binding proteins, clumping factor and collagen-binding proteins. *S. aureus* is a pyogenic bacterium capable of inducing abscess formation in the skin, muscles, urogenital tract, central nervous system and internal organs. Predisposing factors for skin and soft tissue infections include skin damage, skin disease, infections and poor personal hygiene. In the last decades, *S. aureus* has been confirmed as one of the leading causes of hospital-acquired (nosocomial) and community-acquired infections [23].

Toxin-mediated *S. aureus* diseases include the scalded-skin syndrome, toxic shock syndrome and food-borne enterotoxiosis. In each of those diseases, toxins act as “superantigens” and cause the release of large amounts of inflammatory mediators, resulting in fever, rash and hypotension [23].

Staphylococcal scalded-skin syndrome (SSSS) affects most frequently newborns and children, causing remarkable blistering on the superficial skin surface caused by exfoliative toxins released by *S. aureus* [24], known as the two epidermolytic exotoxins A and B [25,26]. The disease is manifested by the formation of watery red blisters on the skin, resulting in a scaled or burned appearance. This infection may lead to the exfoliation of most of the skin surface, followed by acute erythematous cellulitis [27].

Toxic shock syndrome (TSS) has first been described in children [28]. Subsequently, TSS has been associated with the use of super-absorbent tampons by menstruating women [29]. It is caused by the production of toxic shock syndrome toxin (TSST-1) and occurs 2–3 days after the onset of the menses, usually without any evidence of other illnesses. In addition to fever
and hypotension, the disease is typically manifested by a diffuse macular rash on the palms and the soles, with subsequent desquamation (1–2 weeks after disease onset) and multisystem involvement [23].

Enterotoxigenic strains of *S. aureus* are involved into outbreaks of the most prevalent food-borne intoxication, known as staphylococcal enterotoxicosis. The food poisoning is attributed to the ingestion of heat-stable staphylococcal exotoxins; 22 types of staphylococcal enterotoxins (A–V) are currently known [30,31]. The disease onset is rapid (1–6 hrs after ingestion of contaminated food) and explosive, fever is absent [23]. The main clinical signs include nausea, abdominal pain, cramps, vomiting and headache. Symptoms resolve spontaneously within 24–48 hours [32].

To avoid the risk of staphylococcal enterotoxicosis and to protect the health of consumers, food business operators need to ensure that foodstuffs comply with the relevant microbiological criteria set out in Commission Regulation No 2073/2005. Food safety criteria define the acceptability of a product or a batch of food products to be placed on the market. In terms of the above-mentioned regulation, a batch of cheeses (particularly those made of raw, unpasteurised milk) has to be tested for the presence of enterotoxins if the numbers of coagulase-positive staphylococci exceed the limit of $10^5$ CFU g$^{-1}$ [33]. Enterotoxins can be produced by viable cells of staphylococci, albeit only at suitable temperatures. Therefore, the maintenance of the cold chain during production, storage and retail sale of finished food products is crucial to ensure food security [34].

The most common *S. aureus* infections in mammals and birds include clinical or subclinical mastitis, suppurative dermatitis, endometritis, synovitis, arthritis, dermatitis, furuncles, pyemia and septicemia [1]. *S. aureus* subsp. *anaerobius* has been recognised as the causal agent of „abscess disease“ in sheep, with clinical symptoms similar to those of caseous lymphadenitis [35].

Human infections with coagulase-positive staphylococci other than *S. aureus* are rare. There are no published cases of *S. delphini* infections in humans [36]. Foissac *et al.* [37] have documented the case of spondylodiscitis and bacteremia caused by *S. hyicus* in an immunocompetent patient. So far, four cases of human *S. intermedius* infections have been identified, three cases were soft tissue and/or bone infections and one case was a urinary tract infection [38]. The first human case of *S. intermedius* infection of a mechanical prosthesis has been reported by Wang *et al.* [36]; the presumed source of infection was the patient’s dog. Dog ownership, as well as diabetes or immunosuppression, may place patients at higher risk [38].

Among zoonotic pathogens, *S. intermedius* is the predominant cause of skin and soft tissue infections in dogs and human infections associated with bite wounds [39,40]. However, there is no true incidence of *S. intermedius* and *S. pseudintermedius* infections, as these species are frequently misidentified as *S. aureus* [36]. *Staphylococcus schleiferi* subsp. *coagulans* has been isolated from
the external auditory meatus of dogs suffering from external otitis [41]; *S. delphini* has been detected in purulent material taken from two dolphins living in an aquarium [42]. The coagulase-variable *S. hyicus* has been identified as the causative agent of skin lesions, osteomyelitis and occasional bovine mastitis [43], skin lesions ("grease heel") in horses [44], osteomyelitis in poultry [45], septic polyarthritis [46], porcine infectious epidermitis, also known as "greasy pig disease" [47], and reproductive failure in sows [48].

The CoNS are major components of the skin, oropharyngeal and vaginal microflora. These species are reported as less virulent than *S. aureus*. However, numerous studies have revealed the presence of some virulence factors among CoNS [49-52], including their ability to produce heat-stable enterotoxins [53,54]. Some CoNS species are capable of causing serious human diseases and nosocomial infections. Primarily, species of CoNS are important and common causes of prosthetic-device infections. As reported, up to 5% of native-valve endocarditis cases have been due to CoNS [23]. In such cases, the most important property of coagulase-negative staphylococci is their ability to form a biofilm on the surfaces of foreign bodies introduced (implanted) into the organism as a protection against the effects of antibacterial drugs and the immune system of the host [55].

The greatest pathogenic potential has been reported for *S. epidermidis*, as this species has been implicated in bacteraemia, osteomyelitis, polyarthritis, peritonitis, urethritis, pyelonephritis, native and prosthetic valve endocarditis as well as infections of permanent pacemakers, cerebrospinal fluid shunts, prosthetic joints and various orthopaedic devices. *Staphylococcus haemolyticus* may also be associated with human septicaemia, peritonitis, urinary tract infections and native valve endocarditis, while *S. lugdunensis* has been implicated in native and prosthetic valve endocarditis, septicaemia, brain abscess, chronic osteoarthritis, infections of soft tissues, bones and catheters, especially in patients with underlaying diseases. Human infections with *S. schleiferi* subsp. *schleiferi* are manifested by osteoarthritis, bacteraemia, wound infections, brain empyema as well as infections associated with a jugular catheter and cranial drain. *Staphylococcus saprophyticus* has been isolated from patients with urinary tract infections (acute cystitis and pyelonephritis), while *S. simulans* has been isolated from those with chronic osteomyelitis. *Staphylococcus hominis* has been implicated in endocarditis, arthritis, peritonitis and septicaemia, *S. capitis* in endocarditis, septicaemia and catheter infections. *Staphylococcus cohnii* has caused urinary tract infections and arthritis, while subspecies of *S. sciuri* have been isolated from wound, skin and soft tissue infections [1].

In production animals, numerous CoNS species (*S. chromogenes, S. epidermidis, S. haemolyticus, S. hominis, S. saprophyticus, S. sciuri, S. simulans, S. warneri* and *S. xylosus*) have been associated with clinical and subclinical bovine mastitis.
[56-61], while *S. sciuri* has been reported to cause fatal exudative epidermitis in piglets [62].

The increasing clinical significance of CoNS indicates that safety hazards associated with their occurrence in food can be higher than previously assumed [63]. Numerous species of CoNS have been isolated from cheeses, cured meats, sausages, smoked fishes [64], sea-water fish [65], chlorinated drinking water [52], wild rabbits [66], wild pheasants [67] and from Nigerian traditional fermented foods [68]. In general, the occurrence of virulence factors and antimicrobial resistance is more frequent in clinically important CoNS isolates. As compared with isolates from drinking water, virulence factors are more diversified in clinical CoNS strains. Through genes encoding virulence factors or resistance to antibiotics, CoNS may significantly increase the pathogenic potential of the normal skin microflora, including pathogenic coagulase-positive staphylococci [52].

### 3.4. MECHANISMS OF ANTIMICROBIAL RESISTANCE IN STAPHYLOCOCCI

The results of some studies have demonstrated that antibiotic resistance in staphylococci is an ancient feature, as resistance genes have been found in bacterial strains isolated from permafrost sediments in the River Lena region in Central Yakutia, Russia [69], which may be up to 3.5 million years old [70].

A wide spectrum of beta-lactam antibiotics is traditionally used for the treatment of staphylococcal infections. The occurrence of MRSA strains has led to the use of other drug alternatives, including vancomycin, linezolid and daptomycin [71].

Currently, antibiotic resistance is a key issue affecting public health [7]. Staphylococci may become resistant *via* the acquisition of antibiotic resistance genes, mediated by transferable genetic elements. Genes encoding antimicrobial resistance can spread horizontally among bacteria, animals and humans [72]. The mechanisms of gene transfer in *S. aureus* strains include conjugation, transduction and transformation [71].

The primary route of gene acquisition in *S. aureus* strains is bacteriophage transduction. *S. aureus* strains usually harbour one to four functional bacteriophages, integrated in their genomes as prophages [73,74]. After induction, the prophage enters the lytic cycle, which leads to the lysis of the host cell. During the lytic cycle, bacterial DNA may be packaged into the phage capsid and, upon release from the host cell, further transferred to another recipient cell. This phenomenon is known as “generalised transduction” [75].
Plasmids of resistance (R-plasmids) can also be transferred by conjugation. The conjugative transfer of plasmid-encoded resistance genes is recognised as the key mechanism of resistance gene dissemination. The conjugative plasmids carry clusters of genes that encode products necessary for plasmid transfer and mobilisation [70].

The last mechanism of resistance gene transfer is the uptake of both chromosomal and plasmid DNA (including the SCCmecII element) by natural transformation [76]. This process requires a series of naturally encoded competence factors in the S. aureus genome. However, there is still no evidence that transformation is a commonly occurring event [70].

A list of antimicrobial resistance genes and mechanisms of resistance in S. aureus has been published by Reygaert [21]. Most clinical S. aureus isolates contain mobile genetic elements (plasmids) in the size range of 1–60 kbp, which transfer antimicrobial resistance [70]. The oligonucleotide primers used for PCR determination of resistance genes in staphylococci have been reported by Strommenger et al. [77] and Emaneini et al. [78]. However, bacteria that give positive results in genotypic tests not always show positive phenotypic expression of resistance to relevant antibiotics. Therefore, traditional phenotypic tests are unlikely to be replaced by molecular methods based on the detection of resistance genes [7].

3.4.1. Resistance to beta-lactams

Beta-lactams were the first antimicrobial agents introduced into clinical practice and are still used in both human and veterinary medicine. Penicillin was successfully used to treat S. aureus infections until 1942, when the penicillin-resistant S. aureus strain first appeared. Nowadays, the resistance of staphylococci to antimicrobial agents is an issue of worldwide concern [79].

Resistance to beta-lactams is provided by beta-lactamase (also known as penicillinase), encoded by the blaZ gene [80]. Beta-lactamases deactivate the molecule’s antibacterial properties by breaking the antibiotics’ structure through hydrolysis of the beta-lactam ring [81]. In general, penicillin-resistant staphylococci are resistant to all penicillinase-labile penicillins, and penicillin-susceptible staphylococci are also susceptible to other beta-lactams used for the treatment of staphylococcal infections [82]. Therefore, new semisynthetic penicillins, resistant to beta-lactamases, have been developed.

Methicillin was introduced in 1960 for the treatment of infections caused by beta-lactamase-producing staphylococci. In 1961, a MRSA has been reported from England and is now a common cause of hospital-acquired infections [83]. Historically, resistance to the penicillinase-stable penicillins has been referred to as “methicillin resistance” or “oxacillin resistance.” Strains of S. aureus that express the mecA gene or another mechanism of methicillin resistance (changes in the affinity of penicillin-binding proteins for oxacillin) are known as MRSA. Oxacillin-resistant staphylococci are resistant to all currently
available beta-lactams, with the exception of the newer cephalosporins with anti-MRSA activity. Therefore, the resistance to a wide range of beta-lactams may be deduced from testing only penicillin and either cefoxitin or oxacillin. Routine testing of other beta-lactam agents that do not show anti-MRSA activity is not advised [82].

Simultaneous resistance to beta-lactam antibiotics (penicillin, methicillin, oxacillin, nafcillin, cloxacillin and dicloxacillin) is known as „intrinsic“ or „methicillin“ resistance. This type of resistance has been observed in both coagulase-positive and coagulase-negative staphylococci and is usually accompanied with resistance to other groups of antibiotics, particularly cephalosporins [84]. Methicillin resistance is mediated by the \textit{mecA} gene, which is carried on a transposon and encodes the synthesis of a novel penicillin-binding protein PBP2a (also called PBP2′), which displays a low affinity for methicillin [85,86]. It is composed of three structural domains: a characteristic N-terminal structure, a transpeptidase domain and a nonbinding domain [87].

Exposure of MRSA to methicillin inactivates the four high-affinity-binding PBPs normally present; PBP-2a takes over the functions of these PBPs, thus permitting the cell to grow [88]. However, some \textit{S. aureus} strains harbouring the \textit{mecA} gene are susceptible to methicillin [89,90]. Expression of \textit{mecA} can be either constitutive or inducible and is modified by the presence of five chromosomal auxiliary genes, \textit{femA} to \textit{femE} (\textit{fem} is the factor essential for the expression of methicillin resistance), which affect different steps in the synthesis of peptidoglycan and regulate the degree of resistance without altering the levels of PBP2a [91]. The \textit{mecA} gene has been cloned and sequenced along with the genes that control its expression, \textit{mecR1} encoding the signal transducer protein MecR1 and \textit{mecI} encoding the repressor protein MecI [92]. Mechanisms of oxacillin resistance other than \textit{mecA} are rare and include \textit{mecC}, a novel \textit{mecA} homologue [93].

The presence of the \textit{mecA} gene has been confirmed in \textit{S. aureus}, \textit{S. epidermidis}, \textit{S. haemolyticus}, \textit{S. saprophyticus} and \textit{S. fleurettii}. This gene is carried by the SCC\textit{mec} staphylococcal cassette chromosome [94]. Various types and subtypes in SCC\textit{mec} are made up of the \textit{mec} gene complex and the \textit{ccr} gene complex, encoding site-specific recombinase(s) for the movement of the element [92].

Based on the degree of homology to the earliest identified \textit{mecA} gene of \textit{S. aureus} N315 strain, four groups of \textit{mecA} homologues have been described so far [87]. The first group of \textit{mecA} gene homologues (\textit{mecA1} allotypes) has approximately 80 % nucleotide sequence identity to \textit{mecA} of N315 and has been identified on the chromosomes of \textit{S. sciuri} subsp. \textit{sciuri}, \textit{S. sciuri} subsp. \textit{rodentius} and \textit{S. sciuri} subsp. \textit{carnaticum} [95-97]. The second group of \textit{mecA} gene homologues (\textit{mecA2} allotypes) has about 90 % nucleotide identity to \textit{mecA} of N315 and has been identified in \textit{S. vitulinus} [98]. The third group of \textit{mecA} gene homologues (\textit{mecB} genes) is located on the chromosome and
Antimicrobial Resistance in Staphylococci

plasmids of *Macrococcus caseolyticus* JCSC5402 and has 62% nucleotide sequence identity to *mecA* of N315 [99]. The fourth group of *mecA* gene homologues (*mecC* genes), most recently identified in *S. aureus* strain LGA251, shows 69% identity to *mecA* of N315 [100,101].

As most *mecA* gene homologues are associated with mobile DNA elements, their occurrence cannot be limited to the genus *Staphylococcus* and is to be expected among other bacterial genera and species [87].

### 3.4.2. Resistance to tetracyclines

Tetracyclines are broad-spectrum bacteriostatic antibiotics widely used in human and veterinary medicine [102]. Therefore, tetracycline resistance is prevalent among bacteria and is encoded by a wide range of determinants [103]. These antimicrobials exhibit activity against numerous Gram-positive and Gram-negative bacteria as well as atypical organisms [104] such as chlamydiae, mycoplasmas, rickettsiae and protozoan parasites [105]. Tetracyclines bind to the bacterial 30S ribosomal subunit and inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome [106].

The following two mechanisms of resistance to tetracyclines have been described in staphylococci [107]:

- energy-dependent active efflux of the antibiotic from bacterial cells, resulting from the acquisition of the plasmid-located structural genes *tetK* [108-110] and *tetL* [78],
- ribosomal protection by elongation factor-like proteins that are encoded by the transposon located or chromosomal *tetM* and *tetO* determinants [111-115].

In *S. aureus*, both active efflux and ribosomal protection have been successfully induced *in vitro* [109,111]. The plasmid pT181, containing 4,437 base pairs (bp), has first been described by Iordanescu in 1976 [116] as the determinant of tetracycline resistance in *S. aureus*. Sequencing of the tetracycline resistance region of pT181 has shown that it contains a single open reading frame of 1,299 nucleotides [108].

While the *tetK* gene encodes monoresistance to tetracycline, the *tetM* gene seems to carry resistance to all drugs of the tetracycline group, including minocycline, a lipophilic analogue of tetracycline. The MRSA isolates typically carry both *tetK* and *tetM* genes [117]. Hydropathy plotting indicates that the *tetK* protein contains 14 transmembrane α-helices [110]. The *tetL* gene is more widespread among streptococci and enterococci [118] and has been confirmed in strains of *S. aureus* that already carry the *tetM* gene [117]. Although the *tetO* determinant can be found in other Gram-positive cocci (streptococci and enterococci), no reports about *tetO*-positive staphylococci are available [107].
In recent years, the use of tetracyclines has been limited because of the emergence of microbial resistance, leading to the development of glycylcyclines [104]. Glycylcyclines display activity against strains expressing a variety of different tet genes, including those that encode ribosomal protection and efflux mechanisms [105]. Glycylcyclines are also active against methicillin-resistant staphylococci [104].

3.4.3. Resistance to macrolides

In 1952, erythromycin was introduced as the first macrolide antibiotic produced by *Saccharopolyspora erythraea*. Within a year, the occurrence of erythromycin-resistant strains of staphylococci has been reported from many countries [119].

Macrolide antibiotics stimulate the dissociation of the peptidyl-tRNA molecule from the ribosomes at the time of elongation, resulting in chain termination and a reversible inhibition of protein synthesis. Post-transcriptional modification of the 23S rRNA by adenine-N6 methyltransferase has been described as the first mechanism of macrolide resistance.

Currently, rRNA methylases are the best studied mechanisms of macrolide resistance. Synthesis of adenine-N6 methyltransferases is encoded by the group of erm (*erythromycin ribosome methylation*) genes. These enzymes methylate a site on the ribosome, and the conformational change results in a decreased ability of macrolides to bind to the ribosome [119-122]. In general, the *erm* genes have low G+C contents (31 to 34 %) [123], and most of them are associated with conjugative or nonconjugative transposons. These can be found on chromosomes or in plasmids and have a wide host range [124,125]. As the modification of the binding site in the 50S ribosomal subunit by methylases overlaps the binding site of the other macrolide antibiotics, it is usually manifested as a simultaneous resistance to macrolides, lincosamides and streptogramin B antibiotics (MLSb) [119-122], as well as to the new ketolide drugs [126].

The genes *ermA, ermC*, encoding erythromycin resistance, and *msrA*, responsible for the synthesis of ATP-binding protein, are predominant macrolide resistance genes in staphylococci [119,120,127,128]. The plasmid-encoded gene *ermC* is most widely distributed in human and animal staphylococci [129].

Two types of expression have been described for the plasmid-encoded *ermC* gene [119,130]. Constitutive expression is associated with three types of mutations in the *ermC* regulatory region, including deletions [131,132], multiple point mutations [133] and tandem duplications in the *ermC* translational attenuator [134-136]. Inducible *ermC* expression requires the presence of a functionally intact regulatory region 5′ for translational attenuation of the *ermC* methylase gene [119,137].
Tandem duplications and deletions of different sizes also account for constitutive \textit{ermA} gene expression in naturally occurring \textit{S. aureus} isolates [138]. Mutations in the \textit{ermA} gene can arise \textit{in vivo} as well as \textit{in vitro} [139]. While the \textit{in vitro}-derived mutations have been described in human isolates [140,141], \textit{in vivo} mutation has been documented in a naturally occurring \textit{S. intermedius} isolate of avian origin [126].

The development of constitutive \textit{ermC} and \textit{ermA} mutants is a rapid and irreversible process. Constitutive mutants can be obtained \textit{in vitro} after overnight cultivation in the presence of non-inducers [119,136,139,140]. Therefore, noninducers (lincomamides or streptogramins) are not recommended for the treatment of infections caused by staphylococci which show an inducible macrolide-lincosamide-streptogramin B resistance phenotype [138].

3.4.4. Resistance to aminoglycosides

Aminoglycosides are broad-spectrum antibiotics that are used for the treatment of \textit{S. aureus} infections in a combination with other synergistic antibiotics such as beta-lactams or vancomycin. Aminoglycoside antibiotics are not metabolised; they are eliminated by glomerular filtration and excreted as active compounds [142].

Upon contact of Gram-positive bacteria with aminoglycosides, the polycationic molecules of antibiotics are bound to anionic sites of teichoic acid and phospholipids present on their cell surface. The binding causes that the divalent cationic cross-bridges between lipopolysaccharide molecules are displaced, resulting in an increased permeability and the penetration of aminoglycoside molecules into the periplasmic space [143].

Various mechanisms of aminoglycoside resistance have been described; however, most of them have developed in Gram-negative bacteria. In staphylococci, enzymatic inactivation by aminoglycoside-modifying enzymes (AME), such as nucleotidyltransferases, phosphotransferases or acetyltransferases, is the most prevalent mechanism of aminoglycoside resistance [142].

The most common AME-encoding genes among \textit{S. aureus} are \textit{aac(6')-le-aph(2'')}, \textit{aph(3')-IIIa} and \textit{ant(4')-Ia}, which can be harboured on plasmid, chromosome or transposable elements [144]. Resistance to aminoglycosides encoded by the \textit{aacA-D} gene is more prevalent among the human-based biotypes, because this gene is usually more diffused in staphylococci of human origin [145].

In \textit{S. aureus}, resistance to gentamicin, kanamycin and tobramycin is mediated by a bi-functional enzyme displaying AAC (6') and APH (2'') activity [146]. The \textit{aac(6')-aph(2'')} gene is usually present in \textit{Tn4001}-like transposons [147]. The \textit{ANT (4')-IA} enzyme inactivates neomycin, kanamycin, tobramycin and
amikacin, while the APH (3')-III enzyme inactivates neomycin [148,149]. Another two enzymes, ANT(9)-Ia and ANT(9)-Ib, have been described to mediate resistance to spectinomycin. The ANT(9)-Ia was first described in *S. aureus* and then also in *Enterococcus avium*, *E. faecalis* and *E. faecium*. The genes coding for these enzymes are known as ant(9)-Ia and ant(9)-Ib, partially making up Tn554 [140,150].

### 3.4.5. Resistance to chloramphenicol

Chloramphenicol, a broad-spectrum antibiotic, has been isolated from *Streptomyces venezuelae* in 1947 [151] as the first natural substance containing a nitro group [152]. The spectrum of chloramphenicol includes not only Gram-positive and Gram-negative aerobic and anaerobic bacteria, but also chlamydiae, mycoplasmas and rickettsiae [153]. However, because of adverse site effects to meat consumers, arising from chloramphenicol residues in carcasses of slaughter animals (dose-independent irreversible aplastic anaemia), the use of chloramphenicol in human and veterinary medicine has been prohibited by the European Union in 1994 and is currently limited to pets and non-food-producing animals [152,154]. The fluoro substitution at C-3 has led to the synthesis of florfenicol, the fluorinated derivative of chloramphenicol, which does not show any adverse site effects [153,155,156]. Both chloramphenicol and florfenicol inhibit bacterial protein synthesis by interacting with the peptidyltransferase centre at the 50S of the ribosomal subunit [152,157].

The most common mechanism of chloramphenicol resistance is the enzymatic inactivation of chloramphenicol (as well as thiamphenicol and azidamfenicol) by chloramphenicol acetyltransferases (CATs). Acetylated chloramphenicol can no longer bind to the 50S subunit of the ribosome [152]. Two structural types of CATs can be distinguished: the classical CATs (type A CATs) and the novel CATs, also known as xenobiotic CATs [158].

In staphylococci, acetyltransferases are encoded by three groups of *cat* genes, A-7, A-8 and A-9, which are located on small multicopy plasmids. The three prototype plasmids include pC194 [159], pC221 [160] and pSCS7 [161]. In addition to chloramphenicol resistance genes, these plasmids can also carry streptomycin resistance [162] or macrolide resistance genes [163]. In some cases, these genes account for a part of multiresistance plasmids [164,165] or conjugative transposons [166]. On the plasmid pSCFS1 from *S. sciuri*, the cfr gene, conferring resistance to both chloramphenicol and florfenicol, has also been detected [167]. The Cfr methylase modifies A2503 in 23S rRNA and, thereby, mediates resistance to numerous unrelated antibiotics, such as phenicols, lincosamides, pleuromutilins, oxazolidinones and streptogramin A, which bind in close proximity to A2503 at the ribosome [152,167-169].
3.4.6. Resistance to glycopeptides

The widespread use of vancomycin in the treatment of MRSA infections has led to a decrease in vancomycin susceptibility worldwide. In May 1996, the first occurrence of a MRSA strain with reduced sensitivity to vancomycin (MIC 8 mg L\(^{-1}\)) has been reported from the Juntendo Hospital in Tokyo (Japan). This strain was isolated from a surgical wound infection that did not respond to vancomycin therapy and became known as “vancomycin-intermediate \textit{S. aureus}” (VISA) [170]. The resistance mechanisms in VISA isolates are less well defined [171]. However, no presence of any imported mobile genetic element has been observed – the increased vancomycin resistance is related to mutations that appear in the invading pathogen during vancomycin therapy \textit{in vivo} [172].

“Hetero-VISA” (hVISA) strains usually have low vancomycin MIC values (3–8 \(\mu g\) ml\(^{-1}\)), show heterogeneous resistance to beta-lactam antibiotics and serve as precursors of the less frequent homogeneously resistant VISA strains [170,173,174]. Mutations described in different VISA isolates cause the transcriptional changes in genes involved in cell wall synthesis [175-181]. The most frequent genetic alterations associated with the VISA phenotype are mutations in the ribosomal gene \textit{rpoB} [182].

In June 2002, the appearance of the first vancomycin-resistant \textit{S. aureus} (VRSA) strain (MIC >128 \(\mu g\) ml\(^{-1}\)) has been reported in the United States in a patient who developed a catheter exit-site infection. The VRSA isolate was susceptible to chloramphenicol, tetracycline, minocycline, linezolid, quinupristin/dalfopristin, and trimethoprim/sulfamethoxazole and contained both \textit{mecA} and \textit{vanA} genes [183,184]. The strain carried plasmid-borne copies of the transposon Tn1546, which alters the cell wall structure and metabolism and is acquired from vancomycin-resistant \textit{Enterococcus faecalis}. Conjugative transfer of the \textit{vanA} gene from vancomycin-resistant enterococci to \textit{S. aureus} has been demonstrated \textit{in vivo} as well as \textit{in vitro}. Enterococci have become important reservoir of resistance genes. As there are no barriers for the transfer of these genes among Gram-positive cocci, dissemination of glycopeptide resistance to other pathogenic bacteria, including staphylococci, has occurred [185]. Although the transfer of erythromycin and chloramphenicol resistance has been confirmed among staphylococci, the transmission of vancomycin resistance was not documented [186].

Vancomycin does not interact with enzymes participating in the biosynthesis of the bacterial cell wall, but forms complexes with peptidoglycan precursors. Vancomycin resistance requires the presence of operons that encode enzymes essential for [187]:

- the synthesis of low-affinity precursors (modifying the vancomycin-binding target) or
• the elimination of the high-affinity precursors produced by the host (removing the vancomycbin-binding target).

Target modification (vanA-type resistance) is mediated by transposon Tn1546, which encodes resistance to vancomycin and teicoplanin [187]. This 11-kb transposon is ranked among very stable genetic elements [188]. It encodes nine polypeptides, which can be assigned to various functional groups [185], and is generally carried by transferable plasmids [189] and sometimes by the host chromosome as a part of larger conjugative elements [190]. VanA is the most frequently encountered type of glycopeptide resistance in enterococci. In 2008, vanA-type resistance in S. aureus has been reported as rare due to inefficient replication of enterococcal plasmids in staphylococci [185]. In contrary, a very high frequency of vancomycin resistance genes (vanA, vanB) has been reported in S. aureus isolates from patients in Shiraz hospitals (south of Iran). The vanA and vanB resistant genes were detected in 34 and 37% of clinical isolates, respectively [191].

The VanB gene clusters are generally transferred between chromosomes by conjugation [192]. Regarding the sequence differences, the vanB gene cluster was divided into the three subtypes vanB1, vanB2 and vanB3 [193,194]. Acquired vanB-type resistance results from the synthesis of peptidoglycan precursors ending in the depsipeptide D-ala-D-lac instead of the dipeptide D-ala-D-ala [195]. The action of vancomycin is based on the high affinity for the d-alanyl-d-alanine (D-ala-D-ala) residue, a component of the bacterial cell wall precursor Lipid II. Alteration of this residue to D-alanyl-D-lactate (D-ala-D-lac) results in a decreased affinity for the antibiotic [188,190].

3.4.7. Resistance to oxazolidinones

Oxazolidinones are synthetic drugs with excellent oral bioavailability and a predominantly bacteriostatic effect against Gram-positive bacteria, including methicillin- and vancomycin-resistant staphylococci and enterococci [196-198]. Linezolid was the first oxazolidinone approved for clinical use. The oxazolidinones inhibit bacterial protein synthesis at a very early stage by binding to the 23S rRNA in the 50S ribosomal subunit [198,199].

Linezolid-resistant S. aureus has first been isolated in 2001 [200]. Mechanisms of linezolid resistance in staphylococci include point mutations in the V domain of the 23S rRNA genes, mutations in ribosomal proteins as well as in vivo acquisition of the cfr gene [201] that encodes adenine methyltransferase responsible for modifying the adenosine position in the 23S rRNA [202]. Because of a unique mechanism of action, a lack of cross-resistance between oxazolidinones and other antimicrobials has been reported [203].

3.4.8. Resistance to lipopeptides

Lipopeptides represent a diverse group of compounds that consist of a peptide core and a lipid tail and possess a wide therapeutic potential. Daptomycin is a
new semi-synthetic cyclic lipopeptide antibiotic derived from *Streptomyces roseosporus* [204] and particularly used in the treatment of infections caused by MRSA or vancomycin-resistant *Enterococcus faecium* [205]. Currently, the mechanisms of daptomycin action and resistance are not completely understood. However, it is generally accepted that daptomycin penetrates the cytoplasmic membrane of Gram-positive bacteria via a calcium-dependent pathway, which results in bacterial cell wall depolarisation and rapid cell death [206].

Resistance of *S. aureus* to daptomycin occurs stepwise and slowly. Decreased susceptibility is associated with mutations in *mprF, yycG, rpoB* and *rpoC* genes [206]. The emergence of daptomycin-resistant *S. aureus* strains is rare and associated with previous vancomycin exposure [207]. The first mechanism of daptomycin resistance in staphylococci is based on the generation of a more positive cell surface charge, preventing the insertion of positively charged daptomycin-calcium by electrostatic repulsion. The second strategy lays in the alteration of the membrane phospholipid composition due to either a decrease in the amount of phosphatidylglycerol available at the membrane interface or changes in membrane fluidity and homeostasis [204].

### 3.5. METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

The MRSA has first been identified in England in 1961 and soon emerged worldwide [208]. Initially, MRSA was mainly a problem in hospital-acquired infections (HA-MRSA). Since the 1990s, MRSA infections have been increasingly reported among populations and are referred to as community-acquired MRSA (CA-MRSA) infection [209,210].

Currently, MRSA has become a serious problem in hospitals as a major nosocomial pathogen responsible for severe morbidity and mortality worldwide. Colonised and infected patients in hospitals and long-term care facilities are the major reservoirs of MRSA, while carriage by the hands of health care workers accounts for the major mechanism for patient-to-patient transmission [211-213]. Thus, hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA) infections spread easily among patients through the hands of the staff and can lead to frequent epidemics [214,215]. Among intensive care unit patients, nasal carriers of *S. aureus* are at higher risk for *S. aureus* bacteraemia than noncarriers. The MRSA colonisation rates in hospitals vary between 10 and 20% [216,217]. Therefore, hospital sewage is the main source of resistant genes for soil bacteria. As reported by Mandal *et al.* [218], most *S. aureus* strains isolated from hospital effluents were resistant to methicillin (MRSA) and tetracycline. Approximately 15% of MRSA strains showed also VanA-type resistance to vancomycin (VRSA).
Pneumonia and bacteraemia account for the majority of serious clinical MRSA infections, but intra-abdominal infections, osteomyelitis, toxic shock syndrome, food poisoning and deep tissue infections are also important clinical diseases. Surgical site infections, superficial, deep and organ space, can also be caused by MRSA. Postoperative infection with MRSA is a serious and significant problem in liver transplants, but also in prosthetic devices such as endovascular implants, orthopaedic devices and sternal infections [219].

Recently, studies have reported the occurrence of “invasive” MRSA infections with an overall in-hospital fatality rate of 13%. This type of infection is defined as a positive MRSA culture from a normally sterile site, such as blood, pleural fluid, peritoneal fluid, cerebral spinal fluid or bone. Most frequently, positive blood cultures associated with bacteraemia have been detected [220-222].

The phenotypic expression of methicillin resistance shows great variability, and the two main resistance phenotypes include homogeneous and heterogeneous resistance. In a heterogeneous population, all the bacterial cells harbour genetic markers responsible for methicillin resistance. However, phenotypic expression occurs only in a small fraction of the population, varying from $10^{-2}$ to $10^{-8}$. In a homogeneous single population of bacterial cells, these are inhibited by high levels of antibiotic concentration; homogeneous resistance is the least frequent phenotype [223-225].

The MRSA is generated when methicillin-susceptible \textit{S. aureus} (MSSA) exogenously acquires a staphylococcal cassette chromosome \textit{mec} (SCC\textit{mec}) [226]. It is assumed that under the selective pressure caused by the extensive use of antibiotics, MRSA clones which carry resistance genes can better adapt to environmental changes. The resistant genes include the \textit{erm} genes (\textit{ermA}, \textit{ermB} and \textit{ermC}) encoding erythromycin resistance, the \textit{tet} genes (\textit{tetK}, \textit{tetL}, \textit{tetM} and \textit{tetO}) encoding tetracycline resistance and the \textit{aac} genes [227], namely \textit{aph}(2), \textit{aph}(3)-III and \textit{ant}(4)-I genes, which encode resistance to aminoglycosides [107,228]. Adwan \textit{et al.} have evaluated nine resistance genes in 55 clinical MRSA isolates belonging to SCC\textit{mec} types II, III, IVa and V. The reported prevalences of \textit{ermA}, \textit{ermC}, \textit{tetK}, \textit{tetM}, \textit{aacA-aphD}, \textit{vatA}, \textit{vatB} and \textit{vatC} genes were 30.9, 74.5, 76.4, 16.4, 74.5, 1.8, 0 and 5.5 %, respectively [229].

In general, MRSA strains causing infections in humans can be divided into three epidemiological classes [230]:

- community-associated (CA)-MRSA,
- hospital-associated (HA)-MRSA,
- livestock-associated (LA)-MRSA.

While CA-MRSA and HA-MRSA strains predominantly affect humans, most MRSA isolates in food-producing animals belong to \textit{spa}-types associated with LA-MRSA. These strains show only limited transmissibility to human populations. However, \textit{spa}-types associated with CA-MRSA and HA-MRSA have also been reported in food-producing animals [230].
The European Food Safety Authority (EFSA) recommends a systematic surveillance of MRSA in humans. Unfortunately, this monitoring is on voluntary base and therefore, only a low number of European countries participated in the monitoring of MRSA in recent years. In 2015, meat from different food-producing animal species showed the presence of MRSA at various levels of prevalence (Table 2).

### Table 2. MRSA in food, 2015 [230]

<table>
<thead>
<tr>
<th>Country</th>
<th>Food category</th>
<th>Description</th>
<th>Sample unit</th>
<th>Number of units tested</th>
<th>Positive for MRSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finland</td>
<td>Meat from pigs</td>
<td>Fresh, retail, survey</td>
<td>Batch</td>
<td>303</td>
<td>9 (3.0 %)</td>
</tr>
<tr>
<td>Germany</td>
<td>Meat from pigs</td>
<td>Carcass, slaughterhouse, monitoring – active</td>
<td>Batch</td>
<td>342</td>
<td>69 (20.2 %)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fresh, retail, monitoring – active</td>
<td>Single</td>
<td>457</td>
<td>60 (13.1 %)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Meat from pigs</td>
<td>Retail, monitoring</td>
<td>Batch</td>
<td>301</td>
<td>2 (0.7 %)</td>
</tr>
<tr>
<td>Spain</td>
<td>Meat from rabbits</td>
<td>Fresh, retail, surveillance</td>
<td>Single</td>
<td>60</td>
<td>5 (8.3 %)</td>
</tr>
</tbody>
</table>

While Switzerland reported \textit{spa}-type \textit{t034} for positive findings, Finland reported the presence of both \textit{spa}-type \textit{t034} in the meat from cattle as well as \textit{spa}-type \textit{t2741} in pig meat. These \textit{spa}-types were associated with MRSA clonal complex (CC) 398, the common LA-MRSA in Europe [230].

As seen in Table 3, the presence of MRSA was detected in various age categories of cattle (calves under 1 year of age, dairy cows and meat production animals) in three countries (Belgium, Switzerland and Norway); MRSA-positive pigs were detected in four European countries (Germany, Norway, Spain and Switzerland).
Table 3. Occurrence of MRSA in healthy food-producing animals, 2015 [230]

<table>
<thead>
<tr>
<th>Country</th>
<th>Animal</th>
<th>Description</th>
<th>Sample unit</th>
<th>Number of units tested</th>
<th>Positive for MRSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Belgium</td>
<td>Calves (under 1 year of age)</td>
<td>Farm, monitoring – active</td>
<td>Holding</td>
<td>147</td>
<td>116 (78.9 %)</td>
</tr>
<tr>
<td></td>
<td>Dairy cows</td>
<td>Farm, monitoring – active</td>
<td>Holding</td>
<td>96</td>
<td>10 (10.4 %)</td>
</tr>
<tr>
<td></td>
<td>Meat production animals</td>
<td>Farm, monitoring – active</td>
<td>Holding</td>
<td>104</td>
<td>16 (15.4 %)</td>
</tr>
<tr>
<td>Norway</td>
<td>Cattle</td>
<td>Farm, control and eradication programmes</td>
<td>Herd</td>
<td>179</td>
<td>1 (0.6 %)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Calves (under 1 year of age)</td>
<td>Slaughterhouse, monitoring</td>
<td>Animal</td>
<td>292</td>
<td>19 (6.5 %)</td>
</tr>
<tr>
<td>Germany</td>
<td>Pigs – breeding animals</td>
<td>Farm, monitoring – active</td>
<td>Herd</td>
<td>342</td>
<td>90 (26.3 %)</td>
</tr>
<tr>
<td></td>
<td>Fattening pigs</td>
<td>Farm, monitoring – active</td>
<td>Herd</td>
<td>332</td>
<td>137 (41.3 %)</td>
</tr>
<tr>
<td>Norway</td>
<td>Pigs</td>
<td>Farm, control and eradication programmes</td>
<td>Herd</td>
<td>821</td>
<td>4 (0.5 %)</td>
</tr>
<tr>
<td>Spain</td>
<td>Fattening pigs</td>
<td>Slaughterhouse, monitoring EFSA spec.</td>
<td>Batch</td>
<td>383</td>
<td>350 (91.4 %)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>Fattening pigs</td>
<td>Slaughterhouse, monitoring</td>
<td>Animal</td>
<td>300</td>
<td>77 (25.7 %)</td>
</tr>
</tbody>
</table>

Several member states have reported data on clinical investigations for MRSA in different kinds of food-producing animals, such as sheep, goats and cattle (Table 4).
Table 4. Occurrence of MRSA in food-producing animals (clinical investigations, 2015) [230]

<table>
<thead>
<tr>
<th>Country</th>
<th>Animals</th>
<th>Description</th>
<th>Number of animals tested</th>
<th>Positive for MRSA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ireland</td>
<td>Dairy cows</td>
<td>Farm</td>
<td>2,784</td>
<td>1 (0.04 %)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>Dairy cows</td>
<td>Farm</td>
<td>1,344</td>
<td>4 (0.30 %)</td>
</tr>
<tr>
<td>Slovakia</td>
<td>Dairy cows</td>
<td>Farm</td>
<td>366</td>
<td>44 (12.00 %)</td>
</tr>
<tr>
<td></td>
<td>Goats</td>
<td>Farm</td>
<td>18</td>
<td>5 (27.80 %)</td>
</tr>
<tr>
<td></td>
<td>Goats (under 1 year of age)</td>
<td>Farm</td>
<td>3</td>
<td>2 (66.70 %)</td>
</tr>
<tr>
<td></td>
<td>Milk ewes</td>
<td>Farm</td>
<td>39</td>
<td>14 (35.90 %)</td>
</tr>
<tr>
<td>Hungary</td>
<td>Pheasants, meat production flocks</td>
<td>Farm</td>
<td>1</td>
<td>1 (100.00%)</td>
</tr>
</tbody>
</table>

In 2015, the Netherlands and Slovakia have also reported data on MRSA in companion animals, such as cats, dogs or horses (Table 5).

Table 5. MRSA in companion animals (clinical investigations, 2015) [230]

<table>
<thead>
<tr>
<th>Country</th>
<th>Animals</th>
<th>Number of animals tested</th>
<th>MRSA-positive animals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Netherlands</td>
<td>Cats (pet animals)</td>
<td>53</td>
<td>53 (100 %)</td>
</tr>
<tr>
<td></td>
<td>Dogs (pet animals)</td>
<td>50</td>
<td>50 (100 %)</td>
</tr>
<tr>
<td></td>
<td>Horses (domestic)</td>
<td>56</td>
<td>56 (100 %)</td>
</tr>
<tr>
<td>Slovakia</td>
<td>Cats (pet animals)</td>
<td>108</td>
<td>18 (16.7 %)</td>
</tr>
<tr>
<td></td>
<td>Dogs (pet animals)</td>
<td>308</td>
<td>64 (20.8 %)</td>
</tr>
<tr>
<td></td>
<td>Horses (domestic)</td>
<td>1</td>
<td>0 (0 %)</td>
</tr>
</tbody>
</table>

Data on the antimicrobial susceptibility of MRSA isolates from foods and animals have only been reported by three countries (Belgium, Finland and Switzerland). The broth dilution method was used to determine the susceptibility of isolates. All MRSA isolates were resistant to penicillin and cefoxitin; almost all the MRSA isolates were resistant to tetracyclines [230].

Among the MRSA isolates from calves under 1 year of age, tested by Belgium and Switzerland, chloramphenicol resistance was observed in 8.6 and 5.3 % of isolates, respectively. Pig MRSA isolates showed resistance to tiamulin and trimethoprim, probably resulting from the relatively common usage of these drugs in pig medicine in many European countries [230].

Resistance to the important antimicrobials vancomycin and linezolid has not been detected in MRSA from animals or meat in 2015 [230].
3.6. FIGHTING STRATEGIES

According to data of the World Health Organization (WHO), in the EU, 25,000 patients (6.25%) die every year as a result of infections caused by resistant bacteria. Globally the estimated number is as high as 700,000. If current infection and resistance trends are not reversed, 10 million deaths per year are projected between 2015 and 2050 throughout the world, mostly in Africa and Asia.

Infections caused by multidrug-resistant bacteria in the EU result in extra health care costs and productivity losses of at least €1.5 billion each year [4].

The Global Action Plan on Antimicrobial Resistance, developed by the WHO, is based on five general strategies [4]:

- to improve the understanding of antimicrobial resistance;
- to strengthen surveillance and research;
- to reduce the incidence of infections;
- to optimise the use of antibiotics;
- to ensure sustainable investment in countering antimicrobial resistance.

To strengthen the standardised global surveillance and research, the WHO supports the collection, analysis and reporting of data on antimicrobial resistance rates at national levels through the Global Antimicrobial Resistance Surveillance System (GLASS), which was developed in 2015 [4].

International standards and guidelines, aimed at the testing of antimicrobial resistance, are prepared by the Clinical and Laboratory Standards Institute (CLSI). The CLSI Subcommittee on Antimicrobial Susceptibility Testing develops new or revised reference methods for antimicrobial susceptibility tests and establishes breakpoints for the results of such tests as well as quality control parameters. This way, the Subcommittee provides useful information for clinicians and supports them in treating patients with the most effective antimicrobials to decrease antimicrobial resistance [82].

In general, microorganisms are categorised, according to minimal inhibitory concentrations (MICs) or zone diameter values, into the following classes: susceptible, susceptible-dose dependent, intermediate, non-susceptible and resistant. Resistant bacteria are defined as those that are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and, therefore, clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies [82].

Interpretative categories, including breakpoints of selected antibiotics for Staphylococcus spp. using the Disk diffusion test [231] and the Broth or Agar dilution tests [232] in accordance with CLSI performance standards for antimicrobial susceptibility testing, are presented in Table 6 [82].
<table>
<thead>
<tr>
<th>Disk Content</th>
<th>MIC Breakpoints (μg ml⁻¹)</th>
<th>Zone Diameter Breakpoints (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10 units</td>
<td>0.12</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>for S. aureus and S. lugdunensis</td>
<td>30 μg cefoxitin</td>
<td>4</td>
</tr>
<tr>
<td>for CoNS except S. lugdunensis and S. pseudintermedius</td>
<td>0.25</td>
<td>–</td>
</tr>
<tr>
<td>for S. pseudintermedius</td>
<td>30 μg cefoxitin</td>
<td>–</td>
</tr>
<tr>
<td>Ceftaroline</td>
<td>30 μg</td>
<td>1</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>2</td>
<td>4-8</td>
</tr>
<tr>
<td>for S. aureus</td>
<td>4</td>
<td>8-16</td>
</tr>
<tr>
<td>for CoNS</td>
<td>1 μg</td>
<td>0.25</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>10 μg</td>
<td>4</td>
</tr>
<tr>
<td>Amikacin</td>
<td>30 μg</td>
<td>16</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 μg</td>
<td>16</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>10 μg</td>
<td>4</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>15 μg</td>
<td>2</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>15 μg</td>
<td>0.5</td>
</tr>
<tr>
<td>Tetracycline and Minocycline</td>
<td>30 μg</td>
<td>4</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5 μg</td>
<td>1</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>5 μg</td>
<td>1</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>2 μg</td>
<td>0.5</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30 μg</td>
<td>8</td>
</tr>
<tr>
<td>Rifampin</td>
<td>5 μg</td>
<td>1</td>
</tr>
<tr>
<td>Quinupristin-dalfopristin</td>
<td>15 μg</td>
<td>1</td>
</tr>
<tr>
<td>Linezolid</td>
<td>30 μg</td>
<td>4</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>250 or 300 μg</td>
<td>256</td>
</tr>
<tr>
<td>Trimetoprim</td>
<td>5 μg</td>
<td>8</td>
</tr>
</tbody>
</table>

R – resistant  I – intermediate  S – sensitive
As demonstrated in Table 6, cefoxitin can be used to predict the presence of mecA-mediated oxacillin resistance in *S. aureus* and *S. lugdunensis*. While the disk diffusion test is preferred for coagulase-negative staphylococci (except *S. lugdunensis*), dilution tests are the preferred methods for coagulase-positive staphylococci and *S. lugdunensis*. Oxacillin is the preferred agent if a penicillinase-stable penicillin is tested. The results of such testing can be applied to other penicillinase-stable penicillins. For staphylococci that test susceptible, aminoglycosides are used only in combination with other active agents that test susceptible [82].

Prevention and control of antimicrobial resistance through optimising the use of antibiotics require changes in human behaviour. People should only use antibiotics when they are prescribed by a health professional, follow advices of a health worker and avoid any use of leftover drugs. The problem of antimicrobial resistance is becoming more significant in countries where antibiotics can be bought without prescription [233]. However, it is better to prevent staphylococcal infections, *e.g.* by avoiding close contact with sick people, by washing hands, by covering the nose and mouth when sneezing and *via* good manufacturing practices in the food industry.

Staphylococci can easily be spread by infected persons, animals or foods of animal origin. The spread is encouraged by inadequate sanitary conditions, inappropriate hand washing techniques or food-handling procedures. Person-to-person transmission is facilitated by close contact. It has been reported that (HA)-MRSA colonisation rates could significantly be reduced (by 66%) by using alcohol-based hand rub as the most effective method of hand hygiene [215]. Staphylococci can be spread through touch between patients and hospital personnel as well as between people and food-producing or companion animals.

To prevent and control the spread of resistance among staphylococci in the agriculture sector, it is necessary to improve biosecurity on farms, to prevent infections through improved hygiene and animal welfare, to use antibiotics only under veterinary supervision, to apply good practices at each step of producing and processing foods from animal sources and to abstain from the use of antibiotics to prevent diseases in healthy animals [233].

In food-producing animals and agricultural workers, staphylococci are present on the skin surface and can be spread between animals and humans through direct contact. Heat processing of food (pasteurization) results in the elimination of most viable bacterial cells, but is not able to destroy persistent genetic material (DNA). When ingested, genes encoding antimicrobial resistance are released to the digestive tract of the consumer and may be acquired by other susceptible bacteria. Thus, fighting strategies in both the primary production and the food-processing industry consist of the prevention
Antimicrobial Resistance in Staphylococci

of staphylococci in raw materials and finished food products rather than in the reduction of their numbers by further heat processing.

Resistance genes can also be disseminated by potable water and wastewaters. In particular, effluences from hospitals may significantly contribute to spreading resistance in the environment. Therefore, sewage treatment should not only devitalize the emerged bacteria, but rather, a complete destruction of DNA is necessary to inactivate genes encoding virulence factors and antimicrobial resistance. As recently reported, DNA degradation begins at 130 °C and continues in a linear manner until complete degradation at around 190 °C. The combination of moist heat and pressure makes the DNA more sensitive and enables degradation at around 90 °C [234].

The occurrence of staphylococci has been observed in samples of drinking water, wastewaters [235] and biofilms from hospital wastewater [236]. The presence of the meca gene has been reported in S. epidermidis, present in chlorinated drinking water [52], as well as in a biofilm from hospital wastewaters [236]. Municipal and swine slaughterhouse wastewaters may also participate in the dissemination of the meca gene and MRSA and pose a health risk not only to workers, but also to the general public [237]. Soil contaminated with wastewater represents a reservoir of resistant genes. Therefore, both water and soil significantly contribute to a rapid increase in antimicrobial resistance throughout the world.

3.7. CONCLUSION

Resistance of staphylococci to antibiotics is a problem of global public health and food security. When exposed to antimicrobials, staphylococci develop various resistance mechanisms that can emerge and spread worldwide. Currently, the phenomenon of antimicrobial resistance can affect any country of the world. Infections caused by multidrug-resistant S. aureus (MRSA) and S. epidermidis (MRSE) strains result in prolonged hospital stays, higher medical costs and increased mortality. Therefore, tackling antimicrobial resistance is a high priority of the World Health Organization. As the resistance is mainly caused by the misuse or overuse of antibiotics, international cooperation across all government sectors (in particular the human health sector, the animal health sector and the agricultural sector) is required. Effective measures must be taken at each level to stop the spread of resistance. To understand the genetic mechanisms of antimicrobial resistance, further studies are required to monitor resistance genes in isolates of staphylococci from food-producing and companion animals, humans, finished food products as well as from the environment.
REFERENCES


Chapter 4

VANCOMYCIN-RESISTANT ENTEROCOCCUS COLONISATION: IS ISOLATION THE ONLY PRECAUTION?

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Chapter 4

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4.1. INTRODUCTION

The World Health Organization’s World Health Day in 2011 highlighted the problems of antibiotic resistance under the title ‘Antibiotic resistance: no action today, no cure tomorrow’. Every year 25,000 people in the European Union die because of serious resistant bacterial infections that are mostly acquired in healthcare settings.

4.2. GENUS DEFINITION

Thiercelin shared his observations of a saprophytic microbe that pullulated in the human gastrointestinal (GI) tract alongside the bacterium coli. He described this microbe as a Gram-positive diplococcus and proposed the name ‘Enterocoque’ to emphasise its morphology and its intestinal origin [1].

The core morphological and physiological features of enterococci are that they are Gram-positive spherical or ovoid cells that are arranged in pairs or chains [1]. They are nonspore-forming facultative anaerobes and obligatory fermentative chemoorganotrophs. They typically have an optimum growth temperature of 35 °C and a growth range between 10 and 45 °C [2,3]. They typically grow in broth containing 6.5 % NaCl and hydrolyse esculin in the presence of 40 % bile salts [4]. They are usually homofermentative and produce lactic acid as the end product of glucose fermentation without producing gas [5,6].

4.3. ENTEROCOCCUS IN THE ENVIRONMENT

The genus Enterococcus belongs to the Enterococcaceae family along with the Atopobacter, Catellicoccus, Melissococcus, Pilibacter, Tetragenococcus and Vagococcus genera [7]. The Enterococcus genus currently consists of 35 recognised species, with eight more species (Enterococcus lactis, Enterococcus plantarum, Enterococcus quebecensis, Enterococcus rivorum, Enterococcus rotai, Enterococcus ureasiticus, Enterococcus ureilyticus, and Enterococcus viikiensis) likely to be added [8-13].

Enterococci are common and commensal members of the gut community in mammals and birds. They also exist in various other extraenteric habitats, such as soil and sediment, beach sand, aquatic and terrestrial vegetation and ambient waters (i.e. rivers, streams and creeks). They may also be found in heterothermic habitats where temperatures are variable, which is in contrast
to the GI tract of warm-blooded animals where the temperature is relatively constant (i.e. the GI tracts of healthy individuals or hospitalised patients) [14]. Enterococci are found at high concentrations (10^4–10^6 bacteria/g wet weight) in human faeces [15].

A number of researchers have shown that enterococci are more prevalent in roof-harvested rainwater than *Escherichia coli*, and enterococci were suggested to be a better indicator in the assessment of faecal contamination [16]. Nevertheless, exposing hospitalised patients to antibiotics results in major modifications of the gut microbiota, which facilitates the colonisation of the GI tract by drug-resistant enterococci [17]. Among the enterococcal species, *E. faecalis* and *E. faecium* are the most commonly encountered species in human faeces [6,18].

Their low-acidification ability, proteolytic and lipolytic activities, carbohydrate metabolism and volatile compound and bacteriocin production ability allow enterococcal strains to be used in starter cultures in food fermentation or even as probiotics. Despite their use in traditionally-fermented products, some enterococcal strains show technological potential, antibiotic resistance and the presence of virulence factors, which hesitate the direct use of these strains in food technology [19].

### 4.4. DRUG RESISTANCE

Antimicrobial resistance (AMR) in enterococci can be divided into two classes: intrinsic resistance and acquired resistance. Intrinsic resistance is caused by either a lack of target sites for the antimicrobial drug or insufficient penetration of the drug into the intracellular target site. In addition to being the leading multidrug-resistant pathogens in hospitals, they also serve as reservoirs and transmit resistance genes to other bacteria. *E. faecium* and *E. faecalis* are the enterococcal species that are most often associated with multidrug-resistant nosocomial infection, and approximately 30 years ago, both species acquired resistance to the important last-line bactericidal drug, vancomycin [20]. Many different transposons and plasmids have been identified in *E. faecalis* and *E. faecium* that confer resistance to a wide variety of antimicrobial drugs, including erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin [21].
4.5. GLYCOPEPTIDE RESISTANCE

Vancomycin, as well as teicoplanin, belong to a group of glycopeptide antimicrobials. These antimicrobials bind with high affinity to the D-alanyl-D-alanine (D-Ala-D-Ala) C-terminus of peptidoglycan pentapeptide precursors and block the addition of pentapeptide precursors by transglycosylation to the nascent peptidoglycan chain, thereby preventing subsequent cross-linking catalysed by transpeptidation [22,23].

The first clinical isolates of vancomycin-resistant enterococci (VRE), both *E. faecium* and *E. faecalis* were detected in Europe in 1986 [24,25]. Since then, VRE have spread rapidly all over the world [26]. In 1988, French researchers discovered that glycopeptide resistance was plasmid-mediated [24]. A few years later, the same group identified that vancomycin resistance was located on a small mobile genetic element designated transposon Tn1546, encoding the vanA phenotype [27]). Tn1546 belongs to the Tn3 family of transposons that do not encode conjugative functions, therefore dissemination of the transposon can only occur after integration into transferable elements such as plasmids and conjugative transposons [21,27]. Furthermore, a second phenotype (vanB) was also identified on a different mobile element, designated transposon Tn1547 [28].

Molecular characterisation of DNA heterogeneity in the vanA gene cluster of Tn1546 in isolates from humans and animals revealed high levels of DNA polymorphisms due to point mutations, deletions and insertions of different sequences (e.g. IS1216V and IS1251). These polymorphisms can be used to study the epidemiology of Tn1546 [29,30]. Identical Tn1546 variants were identified among vancomycin-resistant *E. faecium* (VREF) from farm animals and humans, which could be the result of colonisation of animal-derived VREF in humans or the transfer of Tn1546 from animal VREF to human enterococcal isolates [31].

4.6. DRUG-RESISTANT ENTEROCOCCI IN NONHUMAN RESERVOIRS

In the environment. Sewage is an important reservoir of resistant enterococci, and tetracycline-resistant enterococci were isolated from sewage as early as the 1970s [32]. Glycopeptide resistance in *E. faecium* was first detected in 1986 in clinical isolates from hospitals in France and the United Kingdom [24,33]. The first indication of a reservoir outside hospitals was the detection of VRE in wastewater treatment plants in small German towns without a hospital and in sewage in the United Kingdom [34,35]. High-level aminoglycoside resistance has been observed globally among enterococci isolated from the environment [36]. More specifically, high-level kanamycin
resistance was observed among 34% of 248 enterococcal isolates from sewage and water in the United States, streptomycin resistance was found in 5% of sewage isolates in the United States and highly gentamicin-resistant enterococci were isolated from sewage and water in the United States and Germany [34,37].

In animals. Enterococcal infections in animals are rarely treated with antimicrobial agents; however, enterococci are exposed to antimicrobial selection in the GI tract during the treatment of other infections or when antibiotics are used as growth promoters or prophylaxis [38]. A multitude of studies have described the occurrence of VRE in nonhuman reservoirs, including cats, dogs, horses, birds, wood frogs, ostriches, pigs, pork, broilers, poultry meat, environmental samples and sewage. VRE were also reported in stool samples from farmers and nonhospitalised humans in the community, mainly in Europe [39]. In certain conditions, feeding animals low doses of antimicrobials can increase productivity by improving feed conversion and decreasing the morbidity and mortality caused by the infection [40]. The glycopeptide, avoparcin, was first introduced for growth promotion in 1975 and was mainly used for broilers, pigs, turkeys, veal calves and other animals [39-42]. As avoparcin confers cross-resistance to vancomycin, its use selected for the growth of VRE, and thus VRE were common in the intestinal flora of farm animals in Europe during the 1990s [43]. In contrast, VRE were not isolated from farm animals in the United States until 2008. Because of this connection, the use of avoparcin as a growth promoter was banned, and the prevalence of VRE in European farm animals rapidly declined but did not disappear [44]. A recent study modelling the persistence of VRE indicated that they will be present among farm animals for a long time, which is in agreement with today's view regarding the timeframe of AMR reversal [45,46].

In food. Antibiotic-resistant enterococci occur in meat products, dairy products and even within strains used as probiotics [47]. Al-Ahmad et al. demonstrated, that after the consumption of cheese, food-borne enterococci can integrate into the oral biofilm in vivo [48]. Thurnheer and Belibasakis confirmed that E. faecalis is able to colonize an in vitro established six-species oral biofilm in high numbers [49]. The finding that E. faecalis from food can incorporate into the oral biofilm and is prevalent in dental diseases raises the question as to whether the oral cavity serves as a reservoir for virulent and resistant strains of E. faecalis [48,50]. The prevalence of antimicrobial-resistant enterococci in food-producing animal is becoming a matter of concern, as these resistant bacteria may be transmitted to humans via the food chain [51]. Tetracycline resistance commonly appears as acquired antimicrobial resistance in Enterococcus [52]. Because tetracycline has been widely used to promote livestock growth and to treat human diseases, the widespread use of this antimicrobial has caused selective pressure and led to an increase in the number of acquired resistance genes among bacteria Enterococci resistant to multiple antimicrobial agents, including vancomycin.
4.7. MICROBIAL COMMUNITIES AND ASSOCIATION WITH AMR

Resistant Genes Exist In Nature. Resistance to commonly-used antibiotics is present in the genes of bacteria everywhere, as researchers at the University of Lyon discovered. A global study of bacterial genomes found resistance across 71 environments, including oceans, soil and human faeces. They found that 30% of all known antibiotic-resistance genes could be found in a single soil sample [53].

It is only in recent years that research regarding antibiotic resistance has focused on the environment from which the antibiotics were initially extracted (i.e. soil microorganisms and the soil ecosystem). With an ever-decreasing supply of novel antibiotics and increasing resistance, emphasis has turned to defining the natural antibiotic resistome and understanding the ecology and evolution of antibiotic resistance in the nonclinical environment [54].

There is evidence that antibiotics have played a role in microbial metabolism for millions of years [55]. Some work by destroying the bacterial cell wall, disorganising the peptidoglycan layer or interrupting enzyme synthesis or signal cascades [56]. In natural environments, certain bacterial species produce their own chemical compounds that are adept at killing other bacteria [57]. This is done to outcompete other species for habitat, nutrients and possible hosts. Antibiotics were seen to control and obliterate fungal and protozoal infections, control crop pests, maintain livestock health and help those with common physiological diseases [58]. However, the environment does not exist in a separate world to that of humans.

There is a constant flow to and from soil, especially in urban and agricultural environments. Human activities such as using antibiotics for the treatment of human and animal diseases and in agriculture, but also pollution and climate change, have altered the soil environment. If the soil is a reservoir of antibiotic-resistance mechanisms, it is important to identify how the actions of humans and climatic change will affect the soil resistome.

Antibiotic resistance in bacteria only leads to a loss of functional systems. Evolution requires a gain of functional systems for bacteria to evolve into man [59]. Therefore antibiotic resistance in bacteria is not an example of evolution in action but rather of variation within a bacterial kind. It is also a testimony to the wonderful design God gave bacteria.

The Habitat. It is estimated that the human body consists of approximately $10^{13}$ cells and hosts $10^{14}$–$10^{15}$ individual microorganisms. These microorganisms can be divided into two groups: 1) those that usually remain constant in their normal habitat (indigenous flora) and 2) those that are accidentally acquired and have to compete with other microorganisms and host defences after their adherence to epithelial or mucosal surfaces.
It is accepted that infection is the result of interaction between the host, the microorganism and the environment. Pathogenicity is not only an intrinsic quality of microorganisms but the consequence of some properties of the microorganisms and the host [60].

Leonard et al. attempted to quantitatively estimate intrinsic pathogenicity in 40 infants admitted to a neonatal surgery unit for at least 5 days. The intrinsic pathogenicity index (IPI) for a species (y) was defined as [61]:

\[
\text{IPI} = \frac{1}{\text{day}^2} \int_0^\text{day} \frac{dN_y}{dt} \ dy
\]

The range of this index is 0–1. The highest IPI of 0.38 was found for Pseudomonas spp., while other isolated potential pathogens had IPI values of <0.1 (i.e. Enterobacter spp. = 0.08, Staphylococcus aureus = 0.06, Klebsiella spp. = 0.05, E. coli = 0.05, Staphylococcus epidermidis = 0.03 and Enterococcus spp. = 0). This index provides useful information about the relative pathogenicity of different microorganisms in a specific population and could be used to design antibiotic policies, both prophylactic and therapeutic, in groups of patients in which microbiological surveillance could be [62].

Interestingly, if the number of bacteria in the human body \((3.9 \times 10^{13})\) is compared to the number of nucleated human cells \((\sim 0.3 \times 10^{13})\), a ratio of approximately 1:10 is obtained [63].

### 4.8. PREVENTING AND CONTROLLING VRE

Risk factors for VRE carriage upon intensive care unit (ICU) admission include the duration of previous hospitalisations, glycopeptide administration, chronic heart failure, malignancy, insulin-dependent diabetes mellitus and previous enterococcal infection (VRE or vancomycin sensitive enterococci). Risk factors for VRE colonisation during an ICU stay include quinolone administration, chronic obstructive pulmonary disease, chronic renal failure and the number of VRE-positive patients in nearby beds [64]. Although the translocation mechanisms of enterococci across the GI tract are uncharacterised, it is known that the overgrowth of VRE in the gut microbiota is an initial condition in the development of VRE infections [17,65,66]. In November 1994, the Hospital Infection Control Practices Advisory Committee ratified a series of recommendations for preventing and controlling the spread of vancomycin resistance, with a special focus on VRE (isolation precautions to prevent patient-to-patient transmission of VRE). These recommendations included placing VRE-infected or colonised patients in private rooms, wearing gloves (clean and nonsterile gloves are adequate) [67].

Preventing colonisation of the upper and lower digestive tract is an approach to prevent ventilator-associated pneumonia (VAP) and other infections. This approach is built on the theory that the GI flora changes with acute illness. In particular, it assumes that the normal flora disappears and is replaced by an overgrowth of potentially-pathogenic microorganisms (PPM). This is followed
by aspiration of PPM, which could finally result in VAP. This review focuses on
the decolonisation methods of VRE without using antibiotics.

4.9. SELECTIVE DECONTAMINATION
OF THE DIGESTIVE TRACT (SDD)

Selective decontamination of the digestive tract (SDD) prevents severe
infections and reduces mortality in critically ill patients. Historical arguments
against its use, such as the development of bacterial resistance and the absence
of influence on mortality, have not been confirmed. Recent clinical trials and
meta-analyses that were designed to evaluate these variables showed
remarkable reductions in the incidence of resistant bacteria and a significant
beneficial effect on survival [68].

SDD consists of the oropharyngeal and gastric application of nonabsorbable
antibiotics (often polymyxin, tobramycin and amphotericin) alongside a short
course of intravenous antibiotics, often cefotaxime [69]. Sanchez-Garcia et al.
demonstrated a reduction in the overall occurrence of nosocomial pneumonia
following the use of SDD; however, the level of carriage of methicillin-resistant
S. aureus and coagulase-negative staphylococci and enterococci was
significantly higher in the SDD-treated patients [70]. SDD and selective
oropharyngeal decontamination (SOD) are not active against resistant Gram-
positive bacteria, therefore they may promote colonisation with bacteria such
as S. aureus and E. faecalis [71]. As a result, SDD does not cover low-level
pathogens, such as anaerobes, viridans streptococci, enterococci and
coagulase-negative staphylococci, which rarely cause infections during an ICU
stay [68].

4.10. PROBIOTICS (BACTERIOTHERAPY AGAINST VRE)

Another strategy uses topically-applied probiotics (live bacteria) that could
alter the GI flora. Recent trials of different probiotic formulas suggest this
strategy is also effective at preventing VAP.

Composed of nearly a thousand different types of microorganisms (some
beneficial, others not), the human gut microbiota plays an important role in
health and disease. The concept of selective decontamination with probiotics,
with or without prebiotics, is at least partly based on colonisation resistance.
Probiotics are live bacteria that could beneficially affect the host by altering the
GI flora, while prebiotics are nondigestible sugars that selectively stimulate the
growth of certain colonic bacteria. When administered in combination,
prebiotics can enhance the survival of probiotic strains as well as stimulate the activity of the endogenous flora. The combination of pre- and probiotics has been termed ‘synbiotics’.

The administration of probiotics is not expected to eradicate PPM in the same way that antibiotics would, but it is expected to delay the time to colonisation while the patients are intubated and ventilated, which would be beneficial. Several probiotic and synbiotic formulas are known and used. They usually contain a combination of lactic acid bacteria (including *Lactobacillus* spp.) and prebiotics or a single-agent probiotic (*Lactobacillus* spp.) [71].

### 4.11. *ENTEROCOCCUS* SPECIES AS PROBIOTICS IN FOODS

*Enterococcus* species are able to produce a wide variety of virulence factors, which highlights their relevance as safety indicators in foods; however, they are also able to produce bacteriocins, called enterocins, and promote specific food modifications during fermentation. Enterococci are present in a variety of ripened cheeses, especially from the Mediterranean region, and are responsible for the specific aromas and flavours that determine the characteristics of these artisan foods. Many *Enterococcus* species were characterised based on their probiotic potential and were included in commercial products to be consumed by humans and animals that aimed to promote health and well-being. Despite being known to possess virulence genes, many studies found the absent expression of such genes, especially in isolates obtained from food systems. This led to studies investigating their real relevance as pathogenic microorganisms [72].

One trial investigated *Lactobacillus rhamnosus* GG (LGG) in the form of commercially-available yoghurt for the treatment of VRE. Subjects were randomly assigned to either the treatment group (receiving 100 g day$^{-1}$ of LGG-containing yoghurt for 4 weeks) or control group (receiving standard pasteurised yoghurt). A total of three faecal samples were obtained at weekly intervals, and treated patients were tested for VRE at 8 weeks. Patients in the control group who failed to clear VRE after 4 weeks were given LGG-containing yoghurt for 4 weeks as an open continuation. Of the 27 enrolled patients, 23 completed the study. Two patients were lost to follow-up, one died and one withdrew. All 11 patients in the treatment group who completed the study were cleared of VRE. This was the first description of a probiotic therapy successfully treating the GI carriage of VRE in renal patients [73]. Szachta et al. used LGG to clear VRE colonisation in hospitalised paediatric patients. A statistically significant difference in the level of VRE-negative children was seen at the end of the third week of the trial, with 62.5% in the probiotic group ($n = 32$) and 24% in the control group ($n = 29$) [74]. Taken together, these studies show the potential of LGG to reduce the level of VRE in colonised
patients, and additional studies on larger cohorts of patients are needed to confirm the anti-VRE effect of LGG. In contrast, *L. rhamnosus* Lcr35, which is related to the GG strain, did not significantly decrease the VRE colonisation density in nine adult patients [75].

The identification of commensal species correlated with a VRE reduction or, for *Vibrio cholerae* and *Clostridium difficile*, supported the idea of using commensal single or mixed strains to stimulate protection against intestinal pathogens and pathobionts [76-78]. Ubeda et al. introduced the notion of commensal key species in VRE clearance. They demonstrated that the reintroduction of the normal intestinal microbiota eliminated VRE from the intestinal tract. VRE clearance was correlated with the presence of *Barnesiella intestihominis*. In addition, analyses of the gut microbiota in patients undergoing allogeneic hematopoietic stem cell transplantation showed that microbiota containing *Barnesiella* correlated with resistance to intestinal domination and infection with VRE. However, it was not proven whether the anti-VRE effect was mediated directly by *B. intestihominis*, indirectly through the host response or was consecutive with the restoration of the microbiota [76]. Therefore its role in colonisation resistance is still unknown.

The only publication reporting a preventive effect of probiotic administration on VRE initial overgrowth used the *Lactococcus lactis* MM19 strain. *L. lactis* MM19 reduced the number of mice harbouring detectable VRE in their microbiota 3 days after inoculation with a vanA-type VRE strain. The authors provided *in vitro* evidence to suggest that the production of the bacteriocin, nisin Z, was responsible for the probiotic effect. In the same study, another bacteriocin-producing bacterium, *Pediococcus acidilactis* MM33, decreased VRE persistence in the mice microbiota 6 days after inoculation, albeit to a lesser extent [79].

A reduction of microbiota diversity decreased the production of RegIIIγ, which promotes VRE overgrowth. Depletion of commensal GI microbiota can be compensated by oral administration of lipopolysaccharides or intraperitoneal injection of flagellin, which restore RegIIIγ expression and reduce VRE density [80,81]. In the future, the use of commensal bacteria will be a tool for the eradication of infective bacteria.

### 4.12. FAECAL TRANSPLANT (FAECAL MICROBIOTA TRANSPLANTATION)

A single course of antibiotics can destroy the good intestinal bacteria. If a person receives a course of antibiotics, there is a good chance that the microorganisms in their digestive tract will become out-of-balance or ruined. This also applies to the eyes, nose, throat, skin, vagina, bladder and other parts
of the body where a bacterial balance is essential for good health. Health professionals usually recommend that probiotics are used in this situation, and sometimes probiotic capsules and pills do help.

In a healthy person, bacteria, yeast, protozoa and other microorganisms are built up over a lifetime. A mother’s milk contains important flora, and during the first few years of life when an infant is crawling around and putting virtually every available object in their mouth, they are building up an essential balance of gut flora that will protect and provide good health for the rest of their lives.

Gaita material was first given in China in the 4th century as ‘yellow soup’ to patients with food poisoning and severe diarrhoea. It was used in the 16th century to treat high fever, pain, vomiting and chronic diarrhoea or constipation, and in the 17th century it was used in veterinary medicine. In the 20th century, ‘warm camel faeces’ was recommended by Bedouins, and during the Second World War, it was used by German soldiers in Africa to verify the cause of the cure. In Anatolia, ‘horse-donkey goat stools’ were used to help recover wounded tissue. In 1958, doctors in Denver used an enema to administer faeces to their patients with fulminant and life-threatening pseudomembranous enterocolitis. The goal of this faecal microbiota transplantation (FMT) was to ‘re-establish the balance of nature’ within the intestinal flora to correct the disruption caused by antibiotic treatment [82].

Faecal material for transplantation can be infused in various ways. The preferred route is colonoscopy; however, transplantation is possible using a nasogastric tube, the nasoduodenal pathway, upper gastrointestinal system (GIS) endoscopy and retention enema [83]. One study investigated the impact of FMT in a cohort of patients with digestive tract colonisation by carbapenem-resistant Enterobacteriaceae (CRE) or VRE. A total of eight patients were included: six carrying CRE and two colonised by VRE. Two patients were free from CRE after 1 month of FMT, and another patient was free from VRE after 3 months [84]. In an industry-sponsored trial using an experimental microbiota suspension, eight of 11 patients (73 %) became VRE negative at 1–6 months after enema-administered FMT; however, the patients may have experienced spontaneous eradication [85].

In conclusion, there has been a substantial increase in CRE and VRE carriage, and every new strategy to decolonise patients should be investigated.

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Vancomycin-resistant enterococcus colonisation: is isolation the only precaution?

Vancomycin-resistant enterococcus colonisation: is isolation the only precaution?


TREATMENT REVIEW OF HOSPITAL ACQUIRED INFECTIONS

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Chapter 5

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5.1. INTRODUCTION

Hospital acquired, or nosocomial, infections are monitored closely by agencies such as the Center for Disease Control and Prevention (CDC) and the U.S. Department of Health and Human Services (HHS) for prevention, control, and improvement of patient safety [1,2]. These infections are associated with approximately two million illnesses, leading to over 95,000 deaths and significant costs of about $33 billion every year [3]. Critically ill patients are at a higher risk for serious infections caused by multi-drug resistant organisms (MDROs). Particularly in the intensive care unit (ICU), nosocomial infections lead to increased length of stay, greater morbidity and mortality rates, and need for post-hospitalization care [4]. Many factors contribute to the higher risks in the ICU including invasive devices, surgical or traumatic wounds, disruption of skin barriers and mucosa, and alterations in adaptive immune responses [4]. Other risk factors include immunosuppression, longer hospital stay, multiple chronic conditions, recent invasive procedures, previous antibiotic use within 90 days, and even receiving care in the critical care units [1]. All hospitals should have an antibiogram readily available so that appropriate empiric therapy can be implemented and to trend patterns of resistance [5,6]. Antimicrobial stewardship is essential in reducing the vastly spreading resistance patterns [7,8]. This chapter will focus on common conditions that occur in critically ill patients, common causative organisms in this population, and evidence-based treatment.

5.2. SEPSIS

Sepsis is preventable yet occurs in more than 1.5 million people in the United States per year, leading to high mortality rates of approximately 250,000 deaths a year [7,9]. Previously known as the presence of infection along with systemic manifestations of an infection, the definition of sepsis has been updated in order for a clear and consistent concept to be understood [10]. Sepsis-1 was often misdiagnosed and improperly treated. According to a study, patients diagnosed with sepsis-1 that were not included in the diagnosis of sepsis-3 had fewer 21-day mortality rates than those diagnosed with sepsis-3 (6.96 % 21-day mortality rate for those meeting sepsis-1 criteria) [11]. In addition, patients diagnosed with sepsis under the sepsis-3 criteria who did not meet the sepsis-1 criteria had a higher mortality rate (10.76 % 21-day mortality rate for those meeting sepsis-3 but not sepsis-1 criteria) [11].

In the newer 2016 definition, or sepsis-3, sepsis is defined as “life-threatening organ dysfunction caused by a dysregulated host response to infection”
Sequential [Sepsis-related] Organ Failure Assessment (SOFA) Score is based on respiration, coagulation, liver, cardiovascular system, central nervous system, and renal function. The risk of mortality increases with higher scores. A quick SOFA (qSOFA) score may be used to promptly identify patients at a greater risk for longer ICU stay or mortality which includes: (1) altered mental status (AMS), (2) systolic blood pressure (SBP) ≤ 100 mm Hg, or (3) respiratory rate ≥ 22 breaths/min [13]. Severe sepsis is no longer considered, and septic shock is defined as “a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality.” Patients with septic shock may have the qSOFA characteristics as well as serum lactate > 2 mmol L⁻¹ and hypotension that requires vasopressors to maintain a mean arterial pressure (MAP) of ≥ 65 mm Hg [13]. In Sepsis 1, Systemic Inflammatory Response Syndrome (SIRS) criteria are ambiguous and do not accurately reflect a dysregulated response due to infection when two or more conditions are met. SIRS consist of temperature > 38 °C or < 36 °C, heart rate (HR) > 90 beats/min, respiratory rate (RR) > 20 breaths/min (or PaCO₂ < 32 mm Hg), and white blood cell (WBC) > 12,000 cells/mm³ or < 4,000 cells/mm³ [13].

As with any other medical emergency, time is an essence and therapy should be initiated as soon as possible to decrease mortality and morbidity. The Surviving Sepsis Campaign of 2016 [14] recommends that in the presence of hypoperfusion, fluid resuscitation with a crystalloid be given at a volume of 30 mL kg⁻¹ within the first 3 h, though controversial. It is imperative to modify the volume of fluid resuscitation in identified patients [15]. Some patients may require more, or less, fluid and should be clinically evaluated based on heart rate, blood pressure, oxygen saturation, urine output, capillary refill, appearance of skin mottling, etc. [14,15]. Crystalloid fluid may be given in 500 mL challenges adding up to an initial resuscitation of 20–30 mL kg⁻¹, measuring responsiveness by an increase in stroke volume [15,16]. Some authors suggest evaluating the inferior vena cava with an ultrasound to assess fluid benefit to help avoid over-resuscitation [15,17]. Lactate may be used as a guide in determining adequate tissue perfusion, though it is not a direct measurement of this marker [14,18]. An important factor in sepsis, as well as antimicrobial stewardship, is to collect appropriate cultures prior to initiation of antimicrobial therapy to prevent sterilization [14]. De-escalating therapy is essential in decreasing resistance, side effects, and costs. In cases where timely collection of specimens is not feasible, it may be more beneficial to administer therapy than to prolong treatment. At least two sets of 20–30 mL of blood per culture, aerobic and anaerobic, should be obtained subsequently within a short interval time due to extreme urgency [14,19]. Once sepsis or septic shock is determined, intravenous (IV) administration of empiric antibiotics is recommended within one hour [14]. Evidence has shown that mortality increases with each additional hour as well as in failure to initiate appropriate empiric therapy to cover all likely pathogens [20-23]. Procalcitonin (PCT)
levels may be used to consider stopping empiric treatment or determining duration. Data has shown the use of PCT helps to reduce treatment duration and aid in early de-escalation for bacterial infections [24-26], however, a meta-analysis did not produce the same evidence of PCT benefit [27]. The vasopressor of choice, when needed, is norepinephrine due to inotropic and vasoconstrictive effects. Norepinephrine is able to increase the MAP with small changes in heart rate and has less tachycardic effects than dopamine.

5.3. HOSPITAL ACQUIRED PNEUMONIA/VENTILATOR ASSOCIATED PNEUMONIA

Hospital acquired pneumonia (HAP) presents as a new lung infiltrate appearing 48 h or longer after being hospitalized, along with clinical signs and symptoms [28]. Clinical presentation includes an onset of a fever, purulent sputum, leukocytosis, and a decline in oxygen levels. Ventilator associated pneumonia (VAP) will appear the same way, however, it occurs 48 h after endotracheal intubation [28]. The Infectious Disease Society of America (IDSA) guidelines for HAP/VAP recognize that there is no gold standard for diagnosis but suggests using non-invasive methods with semiquantitative cultures instead of invasive methods. They do recognize, however, that this is a “weak recommendation” with “low-quality evidence” [28]. The risk factor for Multi Drug Resistance Organisms (MDROs) in HAP consists of the use of intravenous antibiotic therapy in the previous 90 days. The following conditions are risk factors concerning MDROs in VAP: intravenous antibiotic use in the previous 90 days [29-31], hospitalization for 5 days or longer before the onset of VAP [30,32-34], septic shock during VAP [31], acute respiratory distress syndrome (ARDS) [29,31] and renal replacement therapy prior to VAP [29]. It is recommended that patients with HAP/VAP receive empiric coverage for Staphylococcus aureus. Methicillin resistant Staphylococcus aureus (MRSA) may be empirically treated in HAP if > 20 % of the local unit isolates are MRSA, or if the local isolates are between 10–20 % for VAP, risk factors for resistance are present, or the patient has a high risk of mortality [28]. Vancomycin and linezolid are the drugs of choice for MRSA pneumonia. Other agents that have also been studied for MRSA include ceftaroline, teicoplanin, telavancin, tedizolid, and tigecycline [35-37]. Patients with HAP/VAP should also receive empiric therapy for Pseudomonas aeruginosa, and other gram-negative bacilli [28]. Empiric coverage for methicillin-sensitive Staphylococcus aureus (MSSA) may include piperacillin-tazobactam, cefepime, levofloxacin, imipenem, or meropenem [28]. Coverage for MSSA when confirmed can include oxacillin, nafcillin, or cefazolin [28]. Treatment of gram negative organisms are a major concern due to their patterns of becoming resistant to almost all considered antibiotics. Common gram-negative pathogens of pneumonia include
For this reason, when > 10% of local gram-negative isolates are resistant to a single agent or the resistant patterns are unknown, or a risk for infections by an MDRO is present, the guidelines suggest double pseudomonal coverage with two antibiotics from different classes [28]. Single gram-negative therapy with an aminoglycoside is not recommended. Two antipseudomonal agents should also be used when structural lung disease is present such as cystic fibrosis or bronchiectasis [39].

Pan-resistant or nearly pan-resistant patterns with Pseudomonas and Acinetobacter are increasing at an alarming rate. Approximately 13% of healthcare-associated Pseudomonas is multidrug resistant, where at least three antibiotic classes are no longer effective, causing more than 6,000 infections a year. At least 63% of Acinetobacter strains are multidrug-resistant and may cause pneumonia in critically ill patients [40]. Preventative strategies play an integral role in slowing or preventing drug resistance and spread. Appropriate initial antibiotics, timing of initiation, and drug concentration are heavily involved in successful treatment and reduction of in-hospital mortality [38]. Standard precautions include hand washing with soap and water or alcohol-based disinfectants prior to entering and upon exiting patients’ rooms, [1] local surveillance of hospital acquired infections with infection control programs, and intervention protocols [41]. Specific methods to prevent VAP include [42]:

- avoiding intubation if possible
- limiting sedation and assessing the sedation need
- early mobility
- minimize pooling of tracheal secretions above the endotracheal tube cuff
- elevate the head of the bed 30°–45°
- maintain ventilator circuits

These practices aid in preventing the cause or worsening of VAP and help to decrease ventilator days, hospital length of stay, costs, and mortality.

### 5.4. CATHETER-ASSOCIATED URINARY TRACT INFECTION

Catheter-associated urinary tract infections (CAUTIs) are defined as the presence of a urinary tract infection (UTI) signs or symptoms in a patient with an indwelling urethral, indwelling suprapubic, or intermittent catheter with 1000 colony forming units (cfu)/mL and one or more isolated bacterial species excreted from a single catheter urine specimen or a midstream voided urine from a patient whose urethral, suprapubic, or condom catheter was removed
Treatment review of hospital acquired infections

within 48 h. UTI symptoms cannot be identified by another source and typically warrants antimicrobial therapy [43]. Signs and symptoms of a CAUTI include fever, rigors, altered mental status (AMS), malaise, lethargy, flank pain, costovertebral angle tenderness, acute hematuria, and pelvic discomfort. This also includes patients whose catheters have been removed within 48 h but still experience dysuria, urgent or frequent urination, or suprapubic pain or tenderness. Spinal cord injury patients with CAUTIs may experience symptoms mentioned above as well as increased spasticity, onset of urinary incontinence, autonomic dysreflexia, autonomic hyperreflexia, and sense of unease or discomfort during urination [44,45].

On average, 15–25% of hospitalized patients receive urinary catheters, and approximately 75% of UTIs are caused by catheters [46]. Indwelling urinary catheters are an independent risk factor for UTIs. The risk of developing CAUTIs is higher with prolonged use of urinary catheters, therefore, it is important to remove them as soon as they are no longer indicated. In the United States, CAUTIs cause up to 40% of all hospital-acquired infections (HAIs) with a risk of developing catheter-associated (CA) bacteriuria increasing by 3–8 % per in situ catheter day [2,44,47,48]. Bacteriurias are the most common causes of gram-negative bacteremias in hospitalized patients and attribute to approximately 15% of nosocomial bacteremias [44,49].

Urinary catheters should only be used when indicated. Incontinence should not be managed with a urinary catheter unless the patient has failed all other methods and is considering using this alternative. Every institutional facility should develop a policy or protocol with acceptable indications for urinary catheters and educate their staff. An order from the provider should be required before a catheter is placed. Catheters are indicated in the following conditions:

- urinary retention that has failed medical management and surgery is not indicated. This includes temporary or long-term drainage [44,50].
- Urinary incontinence where behavioral and pharmacological management or other less invasive interventions, such as incontinence pads or external collecting devices, are not effective or appropriate. Catheters can also be used in terminally ill patients with urine incontinence for comfort measures [44,50].
- When exact urine output observations are needed [44,50].
- If a patient is unwilling or physically unable to collect urine such as during extended surgical procedures or a genitourinary procedure [44,50].

Urinary catheters should be removed as soon as they are no longer indicated to help reduce CA-bacteriuria and antimicrobial use. Institutions should also implement alert systems with possible automatic stop-orders to decrease unnecessary catheterization. While it is imperative to practice preventative
strategies in efforts to avoid causing a CAUTI, it is impossible to avoid them especially in patients who need long-term catheterization [44].

Mostly all patients with an indwelling catheter will be bacteriuric by 30 days. Short term catheterization is < 30 days while long term is > 30 days. The longer a patient is catheterized, the more likely they are to experience complications such as bacteriuria, bacteremia, catheter obstruction, stone formation, infections, fistula, incontinence, and bladder cancer [51]. Other factors that contribute to the development of CA-bacteriuria are the female sex, older age, diabetes mellitus, no usage of antimicrobials, bacterial growth in the drainage bag or urethral meatus cultures, non-sterile catheter insertion, lack of perineal care, increased serum concentration when catheter is inserted, and deadly underlying diseases [52-54].

Catheter-associated asymptomatic bacteriuria (CA-ASB) is defined as bacteria being present in the urine while having no urinary tract signs or symptoms and typically does not require treatment [43]. Infectious Diseases Society of America (IDSA) adds that this includes patients with 100,000 colony forming units (cfu)/mL and one or more urine bacterial species and have an indwelling urethral, indwelling suprapubic, intermittent catheterization, or a man with a newly placed condom catheter in a single catheter urine specimen. These patients should not be screened or treated unless they are pregnant women or being evaluated for research [44].

The term CA-bacteriuria does not have a true definition. It is often used when a clear differentiation between CAUTI and CA-ASB is not made, which most of the time is CA-ASB. Antimicrobials are often inappropriately used in these patients and contribute to the growing number of resistant organisms [44]. In a freshly placed catheter, colony counts of 100 cfu mL\(^{-1}\) can be considered bacteriuria because this is highly representative of true bladder bacteriuria compared to a voided specimen. Lower colony counts are acceptable in catheter urine samples since they are not as frequently contaminated by periurethral flora like voided urine cultures. If symptoms persist during CA-bacteriuria that is non-contributable to another condition, it may be reasonable to treat the patient and monitor symptoms [44].

Pyuria, the presence of white blood cells in the urine, is not diagnostic of CA-bacteriuria or CAUTI and should not be used to distinguish the two or initiate antimicrobial therapy. However, if pyuria is not present in a symptomatic patient, diagnoses other than CAUTIs should be considered. In addition, malodor or cloudy urine cultures alone should not be used to acquire a urine sample, initiate antimicrobial therapy, or characterize CAUTI from CA-ASB [44].

Naturally when a catheter is inserted, host defense mechanisms are disrupted and easier access for inoculation to the surface permits colonization with uropathogens [43,44]. Significant residual urine in the bladder below the catheter promotes true infection [43,55]. Replicating bacteria can form
biofilms that eventually become polymicrobial and lead to antimicrobial resistance, especially in long-term catheters. Biofilms can form catheter encrustations that may obstruct catheter urine flow. Encrustations are usually produced by *Proteus* species, *Morganella morganii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Providencia stuartii*. These organisms can hydrolyze urine urea to free ammonia, causing an increase in pH aiding in the precipitation of minerals [54].

CAUTIs are frequently polymicrobial and caused by multidrug-resistant (MDR) uropathogens. Patients with short-term catheters who develop bacteriuria are normally affected by a single pathogen [56]. *Escherichia coli* is the most common organism isolated, although other major species such as *Klebsiella*, *Serratia*, *Citrobacter*, *Enterobacter*, *Candida*, *Enterococci*, and *P. aeruginosa*, are also isolated [57,58].

The majority of catheters are made from latex, however, use of silicone-based catheters is increasing due to the number of patients with latex allergies and the incidence of inflammation, toxicity, urethritis, and encrustations with latex-based catheters [59,60].

The concentration of organisms increase the longer an indwelling catheter is in place and decrease significantly when a new catheter is inserted [61]. In short-term catheterization, it is recommended for cultures to be obtained by retrieving a sample from the catheter port using aseptic technique or by piercing the tubing with a needle and syringe if a port is not being used [57]. For long-term indwelling catheters, it is preferred that cultures be obtained from a freshly replaced catheter. This should be done prior to administration of antimicrobial therapy in symptomatic patients [62]. Specimen should not be collected from the drainage bag.

Routine prophylactic treatment for CAUTIs is not recommended due to increased risk of antimicrobial resistance. For a presumed CAUTI, urine culture should be collected after replacement with a fresh catheter but prior to administering empiric antimicrobial therapy [44]. If the catheter is no longer indicated, a specimen from voided midstream urine should be acquired before antimicrobial therapy. Treatment should be streamlined according to cultures and susceptibilities [63]. If a catheter placed over 2 weeks ago is still indicated, it should be replaced at the onset of CAUTI suspicion to decrease symptoms and reduce further CA-bacteriuria.

CAUTIs should be treated with appropriate antimicrobials for 5–7 days if the patient has mild symptoms and is not severely ill [44]. For a patient with a severe infection, it is recommended to treat for 10–14 days even if the patient is no longer catheterized. A 3-day regimen may be considered in women aged 65 or less if there are no signs of upper urinary tract infection once the catheter is removed. The local antibiogram should be used to guide empiric therapy. If fever and other clinical symptoms persist after 72 h, urology may
need to perform further evaluation and a longer duration of treatment may be needed [44].

5.5. CENTRAL LINE-ASSOCIATED BLOOD STREAM INFECTION

Intravascular devices are commonly used for various reasons including administration of IV fluids, hemodialysis, medication administration, and more [64]. These devices can cause infections referred to as central line-associated bloodstream infections (CLABSIs) and are known to increase hospital length of stay (LOS) by 10–20 days and expand costs by up to additional $4,000–$56,000 [65,66]. In the United States, there are more than 250,000 CLABSIs every year while 80,000 occur in the ICUs [67]. CLABSIs, along with VAP, are associated with the greatest amount of deaths due to healthcare [41]. Various types of catheters are listed in Table 1.

<table>
<thead>
<tr>
<th>Table 1: Types of catheters [68]</th>
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<tr>
<td>Peripheral venous catheter</td>
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<td>peripheral arterial catheter</td>
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<td>Midline catheter</td>
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<td>Short-term CVC</td>
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<tr>
<td>pulmonary arterial catheter</td>
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<tr>
<td>Pressure-monitoring system</td>
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<tr>
<td>Peripherally inserted central catheter (PICC)</td>
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<tr>
<td>Long-term CVC</td>
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<tr>
<td>Totally implantable device</td>
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CVC: Central Venous Catheter

Symptoms of an infection may include fever, chills, unexplained hypotension, onset of altered mental status, or clinical signs around the infection site such as inflammation or purulence [69]. Positive blood cultures are also a sign of CLABSI when other sources cannot be identified. If symptoms subside after removal of the catheter within 24 h, this is also a sign of a possible CLABSI but is not an absolute diagnosis [64,70,71]. Developing an infection in any of these devices depends on many factors: The sterility and technique upon installation, type of intravascular device used, purpose of the catheter, insertion site, how often the device is used, length of time the catheter is in place, patient characteristics, and care to avoid causing an infection [68,72]. Processes should be standardized so that maximal safety precaution will be performed such as good hand hygiene, use of chlorhexidine in alcohol when preparing the skin, proper insertion and care techniques, reporting of CLABSI rates, and assessment and re-education when needed [41].
For short-term catheters, < 14 days, the roll plate (semiquantitative) technique for tip cultures should be used, whereby the catheter tip is rolled over an agar plate [73]. In pulmonary artery catheters, cultures should be collected from the introducer tip if infection is suspected [74]. These catheters are more likely to be externally colonized with skin flora.

If an infection is suspected of a long-term catheter, meaning it has been in place for 14 days or more, intraluminal pathogens are likely the cause. Culture samples should be taken from both the insertion site and hub. Semiquantitative growth of > 15 cfus/plate with the same organism strongly suggests bacteremia from the catheter. When suspicious of an infection, remove venous access subcutaneous ports and the tip to send to the microbiology lab for qualitative cultures [75]. The most common afflicting pathogens include coagulase-negative staphylococci, *S. aureus*, *Candida* species, and enteric gram-negative bacilli. Surgically implanted catheters and peripheral central venous catheters (CVCs) may also be infected by *P. aeruginosa* [76,77].

Catheter cultures should not be collected on a routine basis. Culture sampling should only be taken when the clinician suspects a CLABSI [19,68]. Colonization is defined as > 15 cfu of a 5-centimeter catheter tip using semiquantitative cultures or > 10² cfu by quantitative cultures (luminal flushing or sonication) [78]. Growth of the same microbe from one percutaneous blood culture as well as the tip of the catheter, or one blood culture from the hub and the other from a peripheral vein is definitive of a CLABSI if criteria of quantitative cultures or differential time to positivity are met [68,78]. Differential time to positivity is defined as microbial growth of a minimum of two hours between the samples collected from the catheter hub and samples collected from the peripheral vein [68,78]. Both blood samples may be taken from two separate catheter lumens if cultures are unable to be obtained from a peripheral vein. When two blood samples are cultured from catheter lumens and the quantitative cfu count is three times greater from the second sample, diagnosis of CLABSI is possible [68,78]. A CLASBI is not diagnosed if colonization of the catheter is positive but the percutaneous culture is negative [69]. Empiric antibiotics should be started after cultures are collected. Once cultures are confirmed, therapy should be optimally streamlined [67,78].

Vancomycin is the drug of choice for empiric therapy in facilities where MRSA infections are common [78,79]. If the vancomycin MIC is > 2 mcg mL⁻¹, other antibiotics, such as daptomycin, should be initiated. The decision to initiate gram-negative coverage should be made based on antibiogram data, severity of disease, and other risk factors. Therapy may include a fourth generation cephalosporin, carbapenem, or β-lactam/β-lactamase combination plus or minus an aminoglycoside [68]. The day of the first negative blood culture should be counted as day one of therapy. Empiric therapy for femoral catheters in critically ill patients should include coverage of gram-negative bacilli and
Candida species. Echinocandins or fluconazole may be used in certain patients [80]. Fluconazole may be an option in patients without azole use in the prior 3 months and *C. krusei* or *C. glabrata* infection risk is unlikely [68]. When the catheter is removed for uncomplicated CLABSIs, therapy for 5–7 days is appropriate. If the catheter is still in place, therapy along with antibiotic lock should be treated for 10–14 days [68,78].

Antibiotic lock should be used as salvage therapy in patients with CLABSI of long-term catheters along with antimicrobial therapy for 7–14 days [81]. Patients with CLABSI of a short-term catheter is likely to have an extraluminal infection, in which case antibiotic locks are ineffective [77,68]. Ideally, the antibiotic lock should be replaced every 24 h, however, in patients with hemodialysis it is acceptable to allow the solution to dwell for no more than 48 h before replacing. If peripheral cultures are negative while catheter collection is positive, antibiotic lock therapy may be used alone for 10–14 days. These solutions are commonly mixed with 50–100 units of heparin or normal saline, an adequate volume to fill the catheter lumen, Vancomycin lock therapy concentrations should be 5 mg mL⁻¹, at least 1000 times higher than the MIC of the pathogenic organism [79].

A transesophageal echocardiogram (TEE) should be performed in 5–7 days of bacteremia onset for patients with persistently positive blood cultures of >72 h after appropriate antibiotics and catheter removal, as well as patients with a prosthetic heart valve, pacemaker, or implantable defibrillator [82]. If diagnosis of infective endocarditis or osteomyelitis is made, duration of therapy should be 6–8 weeks.

### 5.6. HOSPITAL ONSET CLOSTRIDIUM DIFFICILE

The CDC recognizes *Clostridium difficile* infections (CDIs) as an urgent threat to antibiotic resistance. Every year, about 500,000 people require care for CDI while mortality rates are roughly 29,000 per year. Roughly $5 billion a year is spent on CDIs [83]. *Clostridium difficile* is a gram-positive spore-forming anaerobic bacillus that will colonize the gastrointestinal tract after normal flora has been altered [84]. This colonization takes place after the organism has been ingested or when exposed to environments with spores such as soil, surfaces with contaminated feces (toilets or bathtubs), dirty hands, etc. [85]. An overgrowth of *C. difficile* will cause the production and release of toxins A and B, thus resulting in inflammation of the intestinal epithelium and diarrhea [86-88]. Toxin B is more potent than toxin A and is essential for virulence [89], while toxin A is responsible for severe inflammation, injury to the epithelium, and fluid secretion [90].

Exposure to spores in healthcare settings in the previous 12 weeks increases the risk of active infection. Asymptomatic colonization from healthcare
exposure has an estimated rate of 26% while non-hospitalized patients are 2% [83]. The greatest risk factor to developing active *C. difficile* infection is recent exposure to antibiotics (usually 8 weeks or less) [91]. Older adults, 65 years and above, taking antibiotics and having frequent visits to healthcare facilities are also at an increased risk [85]. Antibiotics most commonly associated with *Clostridium difficile* associated diarrhea (CDAD) are clindamycin, fluoroquinolones, third and fourth generation cephalosporins, and carbapenems [92,93]. Antimicrobial stewardship plays an important role in minimizing the use of antibiotics. Other risk factors for developing CDIs include immunosuppression (chemotherapy, human immunodeficiency virus [HIV] infection, etc.) [94-96], gastrointestinal manipulation including surgery or tube feeding [97], and gastric acid-suppressing medications, such as histamine-2 blockers and proton pump inhibitors [92,98].

Hospital onset or acquired *C. difficile* infection (HO-CDI or HA-CDI) is defined as CDI that tested positive more than three days after hospital admission [83,99]. CDI may be suspected if a person experiences signs and symptoms of watery diarrhea of 3 or more episodes within 24 h that is not attributable to other causes such as laxatives inflammatory bowel disease, enteral feeding, or cancer chemotherapy [93]. Diarrhea may also be accompanied by symptoms of fever, loss of appetite, nausea, and abdominal pain or discomfort [85]. Complications of CDI may consist of electrolyte imbalances, dehydration, hypoalbuminemia, toxic megacolon, bowel perforation, hypotension, renal failure, SIRS, sepsis, and death [100-102]. An active infection of *C. difficile* is confirmed by watery, loose, or unformed stool testing positive for *C. difficile* toxins [103], or radiologic findings indicating pseudomembranous colitis. When testing loose stools is not possible, for instance, in cases of suspected *C. difficile* due to ileus, swabbed specimens or formed stool may be acceptable. Testing on asymptomatic patients or to confirm cure of infection is not recommended. Routine testing is also not recommended due to false positive results and increased costs [104]. Methods of confirmatory testing include enzyme immunoassay (EIA), glutamate dehydrogenase (GDH), toxin testing, or nucleic acid amplification testing (NAAT).

Severity of CDI is defined by the guidelines and other literature per the chart below (Table 2) [102,105,106]. Others may choose to use a scoring system to define the severity of infection [107-109].

| **Table 2**: Severity of *Clostridium difficile* infection |
|-----------------------------------|-----------------------------------|-----------------------------------|
| **Non-severe**                     | **Severe**                        | **Fulminant**                     |
| WBC < 15 cells/mL                  | WBC > 15 cells/mL                 | – Hypotension                     |
| SCr < 1.5                          | SCr > 1.5 x baseline              | – Shock                           |
|                                   |                                   | – Ileus                           |
|                                   |                                   | – Megacolon                       |
To help prevent the spread of *Clostridium difficile* infection, protective equipment such as gloves and gowns worn by any person entering the room of a patient with an active CDI is recommended. These contact precautions along with single rooms are effective infection control methods. Good hand hygiene should also be practiced to prevent the spread of nosocomial infections. With CDI in particular, hands should be cleansed with soap and water instead of alcohol-based disinfectants. Even though clinical evidence has not shown alcohol to affect the occurrence of CDI, spore forming *C. difficile* spores are known to be resistant to alcohol [110,111]. Chlorine products, or other sporicidals, are useful in preventing the spread of CDI in contaminated environments [105].

Upon confirming *C. difficile* infection, discontinue any provoking therapy if possible to decrease the risk of recurrence. A newly diagnosed mild to severe CDI may be treated with vancomycin 125 mg by mouth 4 times a day for 10 days or fidaxomicin 200 mg twice daily for 10 days [106]. Metronidazole 500 mg by mouth or intravenously three times daily for 10 days may be used as an alternative choice for an initial, non-severe episode if the other agents are limited. For severely complicated or fulminant CDI, vancomycin 500 mg by mouth 4 times daily plus or minus metronidazole 500 mg three times daily for 10 days is the treatment of choice [106]. If ileus is present, a vancomycin retention enema of 500 mg in about 100 mL of normal saline every 6 h and metronidazole 500 mg IV every eight hours is recommended. A subtotal colectomy preserving the rectum should be performed if surgical management is necessary. Diverting loop ileostomy with colonic lavage preceding vancomycin flushes may be used as an alternative [106]. If patients have a delayed response to any treatment, an extension to 14 days of therapy may be considered [106]. When waiting for final culture specimen results, empiric treatment may be initiated if suspicion for CDI is high [112]. If a patient experiences a recurrent CDI episode, the first recurrence can be treated with a 10-day course of vancomycin or fidaxomicin if metronidazole was used for the initial episode [106]. The use of metronidazole is not recommended in recurrent episodes due to the increased risk of neurotoxicity with cumulative levels. If standard vancomycin therapy was used to treat the primary episode, the second episode should be treated with a pulse or tapered dose of oral vancomycin, or fidaxomicin. When two or more recurrences have occurred, appropriate therapies include pulse or tapered oral vancomycin, a standard course of oral vancomycin followed by rifaximin, or fidaxomicin. Fecal microbiota transplant (FMT) may also be considered after 2 recurrences (or 3 total episodes) of CDI that were appropriately treated with antibiotics [106]. FMT should be collected from healthy donors and may be administered from a fresh or frozen fecal suspension via nasoduodenal tube or colonoscopy [113,114]. Other therapies that have been studied for CDI include nitazoxanide, tolevamer [115], bezlotoxumab [116], and C. diff toxoid vaccinations [117]. Bezlotoxumab 10 mg kg\(^{-1}\) IV is now approved as an
adjunctive therapy with antibiotics in patients who are at high risk of recurrent CDI. The use of probiotics are not recommended by the Society for Healthcare Epidemiology of America/Infectious Diseases Society of America (SHEA/IDSA) guidelines for primary prevention of CDI, however, there is some evidence of a reduction in both initial and recurrent CDI [106,118-120].

5.7. CONCLUSION

Practices of antimicrobial stewardship are essential in helping to prevent the spread of resistance. Healthcare facilities should implement infection control programs, policies, and stewardship to help prevent the spread of resistance. Hand washing is still the cornerstone in prevention of infections and must not be underestimated or omitted by healthcare professionals. Holding work peers accountable, a well-structured plan, and close surveillance of processes help to reduce hospital acquired infections and the spread of other illnesses. Education and positive feedback from management motivates healthcare workers to utilize safe work practices and adhere to protocols. Barriers to implementation may be due to the absence of leadership, resources, and lack of sense of responsibility. Other factors may include staffing and workload. Programs to reduce HAI are highly encouraged to promote communication, teamwork, and revealing and changing unsafe practices. All healthcare workers should be active in infection prevention and control for the entire organization to promote safety for the patients as well as all employees. Guidelines and evidence-based practices should be used to guide institutional policies and should frequently be reviewed and updated when necessary. Implementing solid practices has shown to decrease mortality and morbidity caused by hospital-acquired infections. We hope that the book chapter provided clear guidance in the aforementioned conditions, will promote safe practices to prevent the cause of infections in institutional settings, bring awareness to, and prolong or stop resistance.

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Treatment review of hospital acquired infections


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NOVEL DRUG DELIVERY SYSTEMS TO COMBAT ANTIMICROBIAL RESISTANCE

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6.1. INTRODUCTION

Although not the first antibiotic discovered, the introduction and development of penicillin to widespread use, changed the face of healthcare and effectively launched an arms race with pathogenic bacteria [1]. Despite the benefits of antibiotics, the evolution and development of antibiotic resistant bacteria have been noted within 20 years of their introduction into therapeutic practice (Table 1). Gram-negative bacterial resistance is of particular concern as there are very few available treatment options following the emergence of resistance.

Table 1: Emergence of resistance against some antibiotics after the start of use (modified from [2])

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Year of introduction as therapeutic</th>
<th>First report of resistance</th>
<th>Time (in years) taken for resistance to occur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>1943 (discovered in 1928)</td>
<td>1940</td>
<td>12 years after discovery but 3 years before the start of mass use [1]</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1950</td>
<td>1959</td>
<td>9</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>1953</td>
<td>1968</td>
<td>15</td>
</tr>
<tr>
<td>Methicillin</td>
<td>1960</td>
<td>1962</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>1967</td>
<td>1979</td>
<td>12</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1972</td>
<td>1988</td>
<td>16</td>
</tr>
<tr>
<td>Imipenem</td>
<td>1985</td>
<td>1998</td>
<td>13</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>1985</td>
<td>1987</td>
<td>2</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>1996</td>
<td>1996</td>
<td>0</td>
</tr>
<tr>
<td>Linezolid</td>
<td>2000</td>
<td>2001</td>
<td>1</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>2003</td>
<td>2005 (rare)</td>
<td>2 [3]</td>
</tr>
<tr>
<td>Ceftaroline</td>
<td>2010</td>
<td>2011</td>
<td>1</td>
</tr>
</tbody>
</table>

The threat of antibiotic resistance has reached alarming proportion with more than 2 million individuals being admitted to the hospital each year in United States, culminating in 23,000 deaths and an overall financial burden reaching 35 billion USD [4]. Unless drastic measures are taken soon, grim predictions estimate the annual worldwide death toll may increase to 10 million per year by 2050 [5]. To exacerbate the situation, the drive towards the development of newer antibiotics has slowed down [6]. The Food and Drug Administration (FDA) approved 49 new antibiotics during 1981–2000 period, out of which 24
were later withdrawn or discontinued, compared to only 16 between 2001–2015 [7]. Worldwide, during the period from 2000–2015 30 new antibiotics were marketed along with two new β-lactam/β-lactamase inhibitor combinations (BBLIC) [6] (Figure 1). Note that β-lactamase will breakdown penicillin and lead to resistance. The drop in approvals may indicate, either a changing, stricter FDA approval process, fewer submissions, or a lack of pharmaceutical industry support for development. Out of the new drugs approved, five were totally new classes of antibiotics, but none were targeted against gram negative bacteria [6].

The overuse and misuse of antibiotics in both healthcare and agriculture, including unnecessary or incorrect prescriptions, poor patient compliance, rapid bacterial evolution in response to bactericidal selective pressure, have led to rising resistance. Improved infectious pathogen diagnostics as well as a relatively dried up discovery and approval pipeline have led to the current crisis [8-10]. In this context, distinctly new approaches to delay or circumvent the emergence of resistance while we await newer antibiotics and novel ways to treat bacterial infections are needed. This chapter will specifically focus on the emerging approaches to combat antibiotic resistance, specifically the development and use of novel drug delivery systems to fight bacterial resistance.

![Figure 1. New antibiotic and BBLIC under development as of 2015](image_url)
6.2. ANTIBIOTIC RESISTANCE

6.2.1. Mechanism of resistance

Although the mechanisms of resistance will not be thoroughly covered as several authors have recently published multiple good reviews [1,2,11,12], a general overview of the topic is provided here to provide context for the intelligent design of novel drug delivery systems to fight resistance. Bacteria can develop antibiotic resistance through a variety of diverse mechanisms. Of these, innate genetic mutations can be seen as a prime cause due to their short generation time and rapid evolution [13]. Mutations that confer resistance provide a fitness advantage and lead to elimination of “less fit”, susceptible strains [13,14]. Resistance via mutation can be considered one form of adaptive resistance. Additionally, adaptive resistance may also be due to environmentally triggered genetic changes such as nutrient conditions, growth state, subtherapeutic concentrations of antibiotics itself [15], etc. Alternatively, bacteria can also gain resistance from other resistant strains via horizontal gene transfer (HGT) (plasmid, transposons, integrons, etc.) during conjugation—“acquired resistance” [15,16].

Regardless of the mechanism, of great concern is the resistance acquired in the presence of an antimicrobial agent. Resistant bacteria can then spread and proliferate under antibiotic selective pressure [17]. Several different genes and pathways may be targeted during resistance acquisition. Specifically, bacteria can modify antibiotics by altering (i) genes involved in the synthesis and positioning of the antibiotic target and the access pathways of the antibiotic into the target. These mutations cause modifications on the cell surface by changing the permeability of the plasma membrane, which leads to reduced entry of the drug into the cell, thereby reducing the interactions between the bacteria and the antibiotic and leading to reduction in the production of entry channels like porins further restricting the entry of the drug inside the cell. (ii) Target protecting genes such as those involved in modification or inactivation of enzymes. Bacteria can synthesize antibiotic modifying enzymes that selectively target and degrade the activity of antibiotics by either hydrolysis, as in the case of β-lactamases, group transfer for aminoglycosides, or redox mechanisms. (iii) Genes involved in changing the cell permeability and modifying the efflux pumps. These pumps are naturally occurring in the bacteria to prevent the accumulation of toxic compounds inside the cell. Upon extended exposure to antibiotics, bacteria can modify these pumps to efflux antibiotics outside the cell. Thus, even the antibiotic reaches inside the cell by escaping other resistant mechanisms, it can still be actively pumped out [15,18,19].

In addition to both direct adaptive and acquired resistance, biofilm formation, where planktonic bacteria irreversibly attach to a surface and are covered by a thick exopolysaccharide (EPS) biopolymer layer made of polysaccharides,
proteins and DNA, represents another important mechanism for the development of resistance. As the biofilm grows in thickness and matures, the bacterial members show maximum resistance to antibiotics [20]. Importantly, while bacteria persist in a biofilm, chronic infection ensues. Alternatively, as soon as the bacteria are redispersed from the biofilm, antibiotic sensitivity is restored. The rapid reversal of resistance from the redispersion of biofilm bacteria suggests an adaptive resistance more than a genetic alteration, although there are elements of genetic alteration as well [21]. Furthermore, several reports have also demonstrated increased mutation frequency in biofilm forming bacteria, which in turn facilitates effective HGT by conjugation and transformation [20,22].

6.2.2. Spread of resistance

Genetically, resistance can spread from one bacteria to another by both "vertical," and "horizontal" methods. During a vertical transfer, parental bacteria pass in antibiotic resistance genes to their progeny. Alternatively, during HGT, bacteria share or exchange genetic material during mating. Importantly, HGT can even occur between different bacterial species. Additionally, bacteriophages may act as vectors for passing resistance traits between bacteria [23]. Apart from vectors, bacteria themselves can also acquire naked DNA from their environment. Furthermore, bacteria move themselves from place to place and spread resistance in the environment.

While the globalization of bacterial species is increasingly common and an important factor for controlling the spread of antibacterial resistance, avoidance of bacterial spread is almost impossible in any environmental setting. Thus, the proper use of antibiotics is essential to prevent further bacterial resistance from emerging. The Centers for Disease Control recently reported in their 2017 report on Antibiotic Use in the United States, that 30 % of antibiotic prescriptions in both the hospital and outpatient settings are not only unnecessary but are also inappropriately prescribed with providers favoring broad-spectrum and last line antibiotics over more targeted, first-line approaches [24]. About 90 % of the antibiotics prescribed are given by general practitioners and most inappropriate use in outpatient settings is prescribing antibiotics for viral infections and respiratory infections [25]. These prescribing patterns also lead to increased risk of side effects [1] and elevated economic burden on the already taxed healthcare system. Nevertheless, we do not find ourselves of the precipice of a return to the pre-antibiotic era of medicine strictly due to prescribers; patients also bear a significant responsibility due to poor compliance, and lack of knowledge that contributes to self-medication, a common practice in developing countries [26]. Irrespective of the type of mechanism of resistance and the spread of resistance, combatting it requires multiple levels of intervention, including new and modified antibiotics, antibiotic conjugates, and cutting edge drug delivery systems, to address critically this rising global problem.
6.3. DELIVERY OF NEW AND MODIFIED ANTIBIOTICS

6.3.1. Antimicrobial peptides
Antimicrobial peptides (AMPs) are a diverse group of molecules that (1) show potent immune modulatory activity and antimicrobial function and (2) are produced by many tissues and cell types in a variety of invertebrate, plant and animal species [27]. AMPs generally consists of 10–50 amino-acid residues, which possess certain common features like amphipathic structure, net positive charge and rapid binding to biological membranes [28]. They are divided into different subcategories based on their structure and amino acid composition and are known to provide broad activity against bacteria, parasites, fungi and viruses. Although their mode of action is still not fully understood, reviews show that cytoplasmic membranes and intracellular molecules are the major targets for AMPs, with the mechanism of action being based on a combination of (1) neutralization of lipopolysaccharide, (2) inhibition of cell wall synthesis, (3) alteration of the membrane potential, and (4) inhibition of proteins involved in cell division [28]. Although resistance to AMPs has been reported, it is very difficult to acquire due to their rapid killing action. AMPs have been shown to be particularly effective delivered in combination with current antibiotics against multi drug resistant (MDR) bacteria [29].

6.3.2. Antibiotic modifications
Altering the antibiotic formulation including (1) targeting them to the site of infection, (2) enhancing selective uptake by bacteria, (3) increasing penetration inside cells to attack infiltrated bacteria, or (4) increasing the local concentration of antibiotic can all be strategies to control and improve antibiotic therapy and thwart the resistance process. Although these strategies have been recognized to improve delivery and efficacy, several methods are available to provide such modifications.

6.3.3. Solubility
Since the introduction of the antibiotic to modern medical practice, several older antibiotics have been abandoned due to toxicity; however, several modifications and new delivery systems may allow for their reintroduction with minimal toxicity. In the early 1980s, the use of polymyxin E (colistin) was limited due to reports of renal toxicity. However, revival of this drug to treat several resistant gram-negative bacterial infections, in less toxic form, may prove to be useful [30]. Hence, colistin sodium found to be less toxic than colistin sulfate. Modifying the solubility of the drug will change its bioavailability thereby altering its toxicity. In the case of poorly soluble antibiotics, modification to a salt form of the drug with an organic counter ion will improve its solubility and bioavailability. Additionally, the solubility of an
antibiotic can be controlled based on pH [31]. However, as a hydrochloride salt form, it shows slow dissolution in stomach at acidic pH due to common ion effect [31]. Ciprofloxacin shows low solubility at neutral pH, leading to low bioavailability. Alternatively, the addition of carboxylic acid salts, oxalate, tartarate, benzoate, malonate, and citrate, to norfloxacin or ciprofloxacin showed higher solubility and faster dissolution at pH 6.4 in pure water and at 6.8 in phosphate buffered saline (PBS), though the opposite effect was observed at an acidic pH of 1.2 [31]. Changing the solubility of the parent compound via addition of an organic counter ion can also stabilize the parent compound, as demonstrated by the improved stability of norfloxacin, which is transformed into dihydrate at similar conditions [32]. Antibiotics with improved stability or enhanced delivery at specific pH have been exploited in a variety of new drug delivery systems [33,34].

6.3.4. Prodrugs

Not only can altering the pH and solubility of a parent compound change its bioavailability but it can also hasten the pharmacokinetics of drug release, necessitating further drug modifications. A polymeric prodrug, which must be metabolized to release the active drug (Figure 2), provides additional control over drug solubility, targeted drug delivery, and antibacterial efficacy and can be an attractive alternative to avoid the rapid development of antibiotic resistance in response to an otherwise ubiquitous antibiotic environment [35].

A polymeric ciprofloxacin prodrug, ciprofloxacin-(phenol)methacrylate (CPM) and PEGMA 950 (O950) conjugate (poly(O950-co-CPM)), made via Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization achieved high drug loading, about 16.5 % wt and showed good in vitro antibacterial activity [35]. CPM also showed very good in vivo activity in a francisella tularensis subsp. novicida (F.t. novicida) mouse infection model with 75 % survival rate, when administered via endotracheal aerosolization, compared 0 % in case of when free ciprofloxacin and slow releasing ciprofloxacin from alkyllic ester conjugate was administered. CPM, with its fast, controlled and sustained delivery kinetics and ability to attack bacteria intracellularly in alveola proved to be lifesaving in the experiment against deadly F.t. novicida infection [36].
Figure 2. Polymeric prodrugs can be cleaved through hydrolysis to provide active drug. Delivery of a prodrug can protect the active drug allowing longer circulation and extended activity.

6.4. USE OF ANTIBIOTIC CONJUGATES

6.4.1. Antibiotic conjugates–hybrid antibiotics

Although the rapid discovery of new antibiotics has slowed to a crawl in the face of rising resistance, a unique way to counter resistance is being explored; conjugating antibiotics with complementary mechanisms to make a hybrid antibiotic is proving to be a value and evolving field of discovery (Figure 3). This approach can be especially effective in combatting MDR bacteria by increasing an antibiotic’s (1) penetration, (2) bioavailability, and (3) spectrum of activity while concurrently reducing its toxicity and risk of resistance through the use of an integrated combination therapy that works synergistically [37]. Similar to the cleavable linkers of prodrugs, two antibiotics with complimentary mechanisms of action can be linked to create a hybrid. Alternatively, a non-cleavable bond may also be used when the hybrid is meant to act as a dual-functioning drug.

Figure 3. Antibiotic Hybrids can be created by using a cleavable or non-cleavable covalent bond to link two drugs with complimentary mechanisms of action together.
Hybrid oxazolidinone–quinolone antibiotics showed improved activity against susceptible as well as resistant gram positive and negative bacteria. While oxazolidinones have limited permeability into gram-positive bacteria, which reduces their ability to reach their RNA target, hybridizing it to a quinolone, which targets DNA topoisomerase/gyrase and has good permeability helps oxazolidinones reach its target, thus improving its efficacy. Oxazolidinones, hybridized with ciprofloxacin, ofloxacin, and levofloxacin respectively via a piperazine group showed high potency against linezolid-susceptible Staphylococcus aureus and Enterococcus faecium, gram positive bacteria, with minimum inhibitory concentration (MIC) ≤ 1 μg mL⁻¹. The hybrids were also active against linezolid and quinolone resistant strains, showing 32–128 fold more activity [38].

While the oxazolidinone–quinolone hybrid provides broad applicability for both gram-positive and gram-negative bacteria, other hybrids are designed to target only specific bacteria, protecting the delicate microbiota balance in body. MBX-500, a hybrid of an anilinuracil, a DNA polymerase inhibitor, and a fluoroquinolone is a unique in that it has selective efficacy against a highly virulent strain of C. difficile but leaves most other gram-negative and gram-positive bacteria unaffected. This targeting ability could render it an antibacterial agent less susceptible to bacterial resistance [39]. Alternatively, other hybrids focus on multivalency, which may improve their affinity towards targeted bacteria. Multivalency can be defined as multiple interaction between ligand and its target, leading to a strong affinity [40,41]. Vancomycin resistant Enterococci (VRE) is a particular threat. Vancomycin and nisin are two different classes of antibiotics, both attacking lipid II of bacterial cell wall, but the target affinity is for two different components of lipid II: for vancomycin, it is tripeptide part (Lys-D-Ala-D-Ala), while for nisin, its N-terminal fragment (residues 1–12) binds to the pyrophosphate part. A hybrid of these two may end up increasing the multivalent affinity toward two different components of lipid II, rendering it a potent drug. The vancomycin-nisin (1–12) hybrid was made through click chemistry. Several different linkers were evaluated, and the hybrid linked by three polyethylene glycol (PEG) units (linker length 12.2 Å) showed the most activity. The hybrid was 40 times more active against VRE when compared to the activity of the individual components. It also showed good activity against gram-negative Klebsiella pneumoniae, against which vancomycin alone is typically ineffective [41].

6.4.2. Nanoparticle/antibiotic conjugates

Another strategy to restore antibacterial activity against resistant bacteria is to conjugate the antibiotic with a nanoparticle. Vancomycin resistant S. aureus (VRSA) has been shown to be 6 times more susceptible to vancomycin conjugated with gold nanoparticles (VGNP) (minimum inhibitory concentration, MIC, is 6 μg mL⁻¹) when compared to unconjugated vancomycin. VGNP is hypothesized to have unspecific binding to transpeptidases in cell wall
of VRSA instead of specific binding to the terminal peptides (d-Lac) of VRSA. Importantly, bacteria with mutated d-Lac are resistant to vancomycin. VGNP also showed activity against *E. coli*. Generally, vancomycin does not show therapeutic activity against gram-negative bacteria due to hindrance of the outer membrane. Despite its larger size, it is hypothesized that VGNP crosses the membrane and destabilizes the lipopolysaccharide membrane, thus allowing vancomycin to bind to the mucopeptide region, causing cell wall lysis. Although this occurs at relatively high MIC (40 μg mL\(^{-1}\)), it is lower than that for unconjugated vancomycin (175 μg mL\(^{-1}\)) [42]. Even though this strategy, in its current form, may not be clinically relevant to treat gram-negative infection, it provides a new route for possible exploration.

While gold nanoparticles may themselves demonstrate antimicrobial activity [43], polyacrylate nanoparticles, which have no known independent antibiotic activity, can be bound to penicillin and penicillin derivatives to restore their activity in methicillin resistant *S. aureus* (MRSA). Penicillin G linked with acrylate via a hydrolysis susceptible covalent bond enhanced activity against MRSA as well as methicillin susceptible *S. aureus* (MSSA) when compared to other formulations. It was 8 times more active against MRSA compared to free penicillin G, having an MIC of 2 μg mL\(^{-1}\). Alternatively, another formulation using an ester linkage between the acrylate particle and penicillin G was not as potent. Non-covalently conjugated, encapsulated drug did not show any notable activity. Covalently bound nanoparticle conjugates such as these have been hypothesized to help the drug to reach the bacterial cell while protecting it from bacterial modifying and inactivating enzymes such as \(\beta\)-lactamase. The release of drug at the site of action may involve hydrolysis and/or esterase activity as well as interaction of nanoparticle with bacterial cell membrane [44].

*Figure 3.* When an antibiotic is delivered with an adjuvant, the adjuvant can bind the \(\beta\)-lactamase, allowing the antibiotic to reach its bacterial target intact and active
6.4.3. Antibiotic adjuvant combinations

While nanoparticle conjugates can be seen as carriers to enhance the activity of their antibiotic partner, adjuvants may also play a critical role in the prevention of bacterial resistance as well as the restoration of antibacterial activity of antibiotics against resistant bacteria. Based on their mechanism, adjuvants can be divided into two main classes. Type I adjuvants block bacterial resistance mechanisms by inhibiting antibiotic degrading enzymes, inactivating efflux pump, etc. Conversely, Type II adjuvants work on the host and potentiate the antibiotic’s action. Only type I adjuvants, which work against β-lactamase and extended-spectrum β-Lactamases (ESBLs), are clinically used. β-lactamase activity plays a crucial role in bacterial resistance against antibiotics containing β-lactam ring [45]. β-lactamase breaks the β-lactam ring open, rendering the antibiotic incapable of its activity. Thus, β-lactamase related resistance is widespread, with more than 1400 β-lactamases identified [46]. β-lactamase inhibitors (BLI) and ESBLs can render penicillins, carbapenems, monobactams and cephalosporins susceptible to bacterial-driven inactivation and resistance. These enzymes can also be secreted from bacterial cells, hydrolyzing the drug even before it reaches the bacteria [47]. In an effort to combat antibiotic inactivating enzymes such as β-lactamase, adjuvants, which first appeared in the 1970s and have little to no independent antibacterial activity, can be delivered with the antibiotic to inhibit and counter enzymes like β-lactamases by irreversibly binding to β-lactamases, protecting the drugs from breaking down [46] (Figure 4). Clavulanic acid, a β-lactamases inhibitor (BLI), was the first adjuvant used in clinical practice [48]. β-lactam/β-lactamases inhibitor combinations (BBLIC) can be powerful weapons against antimicrobial resistance.

Studies showed a decreased rate of resistance emergence when BBLICs were used when compared to antibiotic only. Additionally, in Taiwan, K pneumoniae bacteraemia treated with piperacillin–tazobactam showed decreased risk of ESBL positive isolates compared to previous exposure to oxyimino-β-lactam. Furthermore, four Australian intensive care units reported lower rates of MRSA and Pseudomonas aeruginosa infection when BBLICs were used in contrast to cefepime treatment. Piperacillin–tazobactam therapy, as opposed to carbapenems, produced a decline in carbapenem-resistant Enterobacteriaceae, in Illinois. In the UK, a change in prescribing habits have trended towards use of BBLICs and away from third-generation cephalosporins, which have led to a decrease in cephalosporin resistance in Enterobacteriaceae isolated from bloodstream infections. The therapy did not produce any increase in piperacillin–tazobactam resistance [49]. In India, Cefepime–tazobactam and ceftiraxone–sulbactam combinations are now available in the market; both showing good in vitro efficacy against in different clinical isolates [49]. Ceftolozane/tazobactam and ceftazidime/avibactam combinations got FDA approval in 2014 and 2015, respectively [7]. Avibactam is a unique and novel BLI. It is a covalent, reversible, non-β-lactam β-lactamase
inhibitor. It is the most potent BLI to date with a 1–5 : 1 ratio of avibactam: β-lactamase needed for inhibition, compared to >50:1, BLI:β-lactamase molecule, for tazabactam and clavulanate [46]. Moreover, ceftolozane/tazobactam have performed well against ESBL producing Enterobacteriaceae and K. pneumoniae infections in clinical studies [46]. Clavulanic acid, tazobactam, sulbactam and avibactam are adjuvants in use. Aspergillomarasmine A (AMA) is a potent metallo-β-lactamase (MBL) inhibitor that recently showed promise in irreversibly binding and inactivating MBL both in vitro and in vivo. When the mice were treated with a meropenem and AMA combination, the survival rate of mice infected with a lethal NDM-1 positive K. pneumoniae load was more than 95% with a single dose compared to 0% in case of meropenem alone [50]. The combination of imipenem/relebactam significantly lowered the MIC and showed synergistic killing of resistant strains of P. aeruginosa and K. pneumoniae in several in vitro studies [46].

6.5. TRADITIONAL AND EMERGING APPROACHES TO COMBATE RESISTANCE–DRUG DELIVERY SYSTEMS

6.5.1. Non-targeted controlled delivery

Alterations in the drug can also change the pharmacokinetics of drug release, which may prove an effective alternative to delay the emergence of antibiotic resistance. Controlled extended release formulations have long been used to treat disease conditions; however, for infectious conditions extended antibiotic release can inadvertently promote the development of bacterial resistance (e.g., antibiotic loaded bone cement). Controlling the pharmacokinetics of local extended release formulations via the physical properties (i.e., solubility, pKa, etc.) of the drug may mitigate this risk while still providing the advantages of local controlled release. Making a hydrophilic antibiotic less soluble can be a viable strategy to extend its release [51]. This phenomenon has been observed with local vancomycin release from a bone graft void filler [52]. The clinical form of vancomycin, vancomycin HCl, was exhausted from its bone graft depot within 2–3 weeks; whereas, release of the desalted form of vancomycin was extended to 7 weeks (unpublished data). Gentamicin sodium bis(2-ethylhexyl)sulfosuccinate (AOT), a less soluble form of gentamicin, in PLGA nanoparticle showed release up to 70 days [52]. Other fatty acid derivatives of gentamicin such as gentamicin sodium dodecyl sulfate, gentamicin laurate and gentamicin palmitate also showed slower release from vascular prostheses [53]. Collagen fleece containing readily soluble gentamicin sulfate and gentamicin crobefate, a hydrophobic modification of gentamicin, showed slow
and sustained release for up to 12 days with initial high concentration due to the hydrophilic part and sustained release due to the hydrophobic form [54].

Of particular concern for extended release formulations in the development of antibiotic resistance is the consistent presence of antibiotic, which can drive bacteria to infiltrate host cells. Thus, treatment of intracellular bacteria must be considered with formulations designed specifically for this challenging case. Phagocytic cells easily take up liposomes, providing a mechanism for intracellular antibiotic drug delivery. Intracellular lethal bacteria, *F. tularensis*, can be treated via liposomal delivery of ciprofloxacin. In a *F. tularensis* mouse infection model, this liposomal ciprofloxacin preparation showed significantly better survival than free ciprofloxacin [55]. Another study with liposome encapsulated ciprofloxacin demonstrated enhanced phagocytic functions of macrophage, in turn showing antibacterial activity against *S. aureus* via two mechanisms: activity due to antibiotic and activity due to increased phagocytic function. By addressing intracellular colonization, not only can primary infection be addressed but also secondary, infectious relapses may be minimized [56].

6.5.2. Infection responsive drug delivery systems

Drug delivery systems that respond to molecular cues unique to tissues or disease conditions have recently gained widespread scientific interest. Many disease conditions have been characterized by upregulation of specific proteases, which can act as therapeutic targets, disease markers, etc. [57]. Several enzymes were reported to be elevated during infection including a thrombin-like enzyme in *S. aureus*, myeloperoxidase in *Candida*, and esterases in uropathogens. These enzymes can be used as biomarkers for timely initiation of treatment and also as therapeutic targets for infection responsive drug release system (Figure 5) [58-59]. *S. aureus* and *P. aeruginosa* infected wound fluid has been reported to have high thrombin-like activity due to bacterial enzymatic activity [60]; whereas, the release of bacterial phosphatase and phospholipase, which are key virulence factors for certain bacteria, are reported in other studies [61]. Likewise, elevated concentrations of myeloperoxidase released from neutrophils and monocytes have been reported during the inflammatory response to infection [62,63], while macrophages produced cholesterol esterase during infection [64]. Infection with intracellular pathogens like *Mycobacterium tuberculosis* was also shown to increase glycolysis, including glycolytic enzymes and glucose transporters, while downregulating tricarboxylic acid cycle, suggesting that immune cells prefer aerobic glycolysis during tuberculosis infection [65]. These unique biological cues can be exploited during the develop of novel, infection-triggered, local drug release systems, which not only increase therapeutic efficacy, but also decrease toxic systemic side effects. Importantly, such drug delivery systems would not necessarily need to be targeted.
6.5.3. Targeted drug delivery systems

*Clostridium difficile* is the most common infectious, diarrhea-causing organism that resides in the human colon. Upon initiation of antibiotic therapy, the normal gut flora was disrupted, enabling *C. difficile* to persist in the gut as the dominant bacteria and induce colitis [66,67]. Keeping in mind that virulence is often tied to resistance [68], the emergence of highly virulent strains of this bacteria and a reduced susceptibility to antibiotics has led to the development of MDR strains [69]. Fortunately, recent studies have shown that certain lytic enzymes from bacteria and bacteriophages can act as highly selective and effective antimicrobials [70]. These enzymes can be delivered without a traditional targeted delivery system and yet still remain selective against a wide range of *C. difficile*, preserving the normal gut flora.

*Chlamydia trachomatis* (CT), which grows in the genital mucosal cells, is the leading cause of sexually transmitted diseases [71]. CT is an intracellular pathogen residing inside the host cell in a protective inclusion body, which has selective interactions with the exocytic pathways [72]. This niche of protective layers limits the accessibility of antibiotics. Recent findings suggest the importance of the transferrin iron pathway as an intracellular drug carrier. Iron containing transferrin is internalized into the endosome of the cell where...
transferrin is gradually acidified to release iron into the cytoplasm [73]. In a similar manner, antibiotics can be loaded into transferrin where it can act as a vehicle to deliver the drug into the cells. Utilizing bacteria’s own iron scavengers and transporters is a promising strategy to combat the resistance of CT to a broad variety of antibiotics.

Similar to CT, tuberculosis remains worldwide public health threat affecting 1% of the total population every year, making it the number one cause of death due to infectious diseases [74]. Additionally, Mycobacterium tuberculosis, is an intracellular pathogen, which can be phagocytosed by alveolar macrophages (AM) where they adapt to the host cell microenvironment to survive for years [75]. One of the prime factors for the survival of M. tuberculosis inside the AM is the specific interaction between the pathogen and the host, which occurs through various cell surface receptors including mannose receptor [76]. The virulent M. tuberculosis has a unique cell wall mannose-capped lipoarabinomannan, which interacts with the mannose receptor on the macrophages to enter into these cells [77,78]. This transporter pathway can be hijacked in a similar fashion by coating drug carrying vehicles with mannose receptor recognition sequences to target AM intracellular bacteria.

6.5.4. Biofilm targeting approaches

No discussion of antibiotic resistance would be complete without considering the unique and challenging case of drug delivery to biofilms. Biofilms are a cluster of microbes grouped together and attached to a surface protected by secreted extra cellular matrix (ECM)/extracellular polymeric substance (EPS). This transition of bacteria from planktonic to sessile occurs due to environmental changes and gene expression changes, which modify the expression of surface molecules, thereby changing the virulence of the bacteria and altering the nutrient utilization of the bacteria [79]. The complex structure of the ECM/EPS and the constant changing behavior of the biofilm architecture makes a biofilm highly tolerant towards antibiotic treatment. At the most rudimentary level, mature biofilms can be disturbed by mechanical means using water sprays and jets along with debridement of surgical site infections [81]. Alternatively, biofilm formation can be targeted at various stages throughout their life cycle [80] (Figure 6).
During the initial phase of biofilm formation, bacteria attaching to the surface can be prevented by targeting cell-surface-associated adhesins (appendages, proteins and EPS). Many bacteria rely heavily on type 1 pili through FimH, curli fibers and Antigen 43 to mediate attachment on abiotic surfaces [79]. Small molecules like peptides and mannosides that have targeted these different adhesins have shown efficacy in prevention and treatment of both bacterial and fungal biofilm infections. Studies reported mannosides targeting the bacterial adhesin FimH prevented catheter-associated urinary tract infection (UTI) in mice [80]. Pharmaceutically active surface coatings have long been investigated to both prevent adhesion to an implanted device and kill planktonic bacteria in the vicinity of the device, thereby reducing the formation of biofilm. However, caution must be exercised when taking this approach due to the persistent presence of antibiotic that may lead to selection of antibiotic resistance in the absence of infection or by inappropriate use of antibiotics [81]. Conversely, the production of EPS and cellular division can be inhibited. The production of EPS involves both extracellular and intracellular signaling networks as well as non-signaling mechanisms. Cyclic-di-GMP (c-di-GMP) and cyclic-di-AMP (c-di-AMP) were found to control various EPS producing exoenzymes, polysaccharides and adhesins, making these molecules potential candidates to inhibit EPS [82,83]. EPS is synthesized by a virulence factor called glucosyltransferases (Gfts) in pathogens like Streptococcus mutans, which forms the glucan layer of the biofilm. Studies showed that using small-molecule inhibitors of glucosyltransferase, there was a decrease in the accumulation of pathogenic biofilms on teeth [84]. These small molecule inhibitors when used in combination with other approaches like low electric fields greatly improve therapeutic efficacy [85,86]. In addition to titanium implant associated biofilm development with a traditional EPS composition, urinary tract infections, which are usually associated with biofilm formation, produce urease to hydrolyze urea to ammonium ions causing urinary
obstruction. The formation of these ions leads to the precipitation of magnesium and calcium phosphate crystals, which form a protective EPS-like layer for the biofilm, while also increasing the pH of the urine [87]. Although not a traditional targeting strategy, specifically inhibiting the enzyme urease to prevent the production of these ions is an important alternative that should be considered in these cases. Other strategies include decreasing the urine pH to inhibit the formation of ions and pH induced delivery of antibiotics during infection. Lastly, dispersion of biofilm, the final stage in a mature biofilm, can be induced via mechanical means as free-floating, planktonic bacteria are more susceptible to therapeutic interventions.

6.6. OTHER EMERGING APPROACHES AND CARRIERS

6.6.1. Bacteriophage

Bacterial resistance to antibiotics as well as their ability to resist the host immune system is interconnected. Since bacteria adapt to the immune system, they become resistant to a spectrum of antibiotics based on the host's exposure history, but this adaptation also makes them susceptible to other novel classes of antimicrobial compounds [88,89]. One of the most promising approaches is the use of engineered bacteriophages to combat resistance [90]. The use of a phage as a nucleic acid drug carrier allows a targeted bacterial infection. Recent advances in CRISPR gene editing allowed the creation of a phage to remove a bacterial resistance gene and eventually kill the bacteria [91]. Nevertheless, relying on natural bacteriophage mechanisms of infection may prove susceptible to bacterial resistance.

6.6.2. Antibiotic drug discovery and development

Although most antibiotics are either carbohydrate based molecules or carbohydrate receptor targeting molecules, new targets and new classes of antibiotics are consistently be sought out and generated. In fact new molecules that mimic critical carbohydrates with improved properties could be a promising approach [92]. Targeting the inhibition of lipopolysaccharide (LPS), specifically the synthesis of the lipid A region, in gram-negative bacteria is such an approach. A more specific strategy to target the core oligosaccharide is the inhibition of enzymes involved in the biosynthesis of O-antigens, specific to different bacterial strains. Another strategy that uses unique cell-surface carbohydrates, is the design of unnatural amino acids like D-peptides, L-DNA, or L-RNA as enzymatic resistant receptors. Transglycolase is one of the key enzymes involved in the polymerization of the disaccharide units of peptidoglycan. This enzyme can be used as a target for small molecule drugs as it is easily accessible due to its location on the cell surface. With the recent
advances in functional genomics, unique RNA sequences will serve as new drug targets. Aminoglycosides, which are primarily involved in the inhibition of protein synthesis in bacteria, may serve as the primary source of small molecules for the identification of new leads to target RNA. However, with each new drug developed a novel, microenvironment triggered or targeted delivery system should be developed in conjunction.

6.7. CONCLUSION

Although antimicrobial therapy has been one of the important advances in modern medicine, antibiotic resistance has quickly developed to become one of the greatest public health threats and identification of pipeline of new antibiotics became more challenging. Development of novel strategies along with sophisticated diagnostic technology is the need of the hour. Drug delivery strategies (Figure 7) based on a combination of infection specific mechanisms targeting represent a promising pathway forward to better utilize our current antibiotic arsenal to combat drug resistance. Although targeted delivery and formulation modifications have been successful in the research to combat resistance, combination therapies with synergistic antibiotic mechanisms and delivery strategies are continuing to gain traction.
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CERAGENINS AS NON-PEPTIDE MIMICS OF ENDOGENOUS ANTIMICROBIAL PEPTIDES

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Chapter 7

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7.1. INTRODUCTION

Antimicrobial peptides (AMPs) are evolutionarily-conserved molecules that are produced as a first line of defense by organisms ranging from prokaryotes to humans. In higher organisms, they are naturally found in the skin, airways, gastrointestinal tract and reproductive tracts with the highest concentrations of AMPs being found in the tissues that are regularly exposed to pathogens. They display broad-spectrum activity against bacteria, fungi, lipid-enveloped viruses and parasites. In higher organisms, AMPs exert additional immunomodulatory activities such as anti-inflammatory properties, through sequestration of bacterial endotoxins, and acceleration of wound healing by promotion of cell migration into wound beds and neovascularization. Common features of AMPs are juxtaposed cationic amino acids and hydrophobic residues on opposing faces of helices or sheets. This morphology provides them with a unique amphiphilic structure that selectively associates with microbial membranes, disrupts membrane integrity, and leads to cell death.

Despite the broad-spectrum antimicrobial activities of AMPs and the belief that microorganisms are unlikely to become resistant to AMPs, their clinical application is limited due to the high cost of production and their degradation by bacterial proteases. In order to overcome these clinical challenges, ceragenins were developed as non-peptide mimics of AMPs. They are based on a common bile acid and mimic the amphiphilic morphology of AMPs with a similar spectrum of activity against bacteria, fungi and lipid-enveloped viruses. Straightforward preparation of ceragenins and their stability in the presence of proteases paves the way for large-scale production and clinical application. This chapter will discuss the development of ceragenins, their breadth of antimicrobial activities, impacts on innate immune functions and wound healing, and specific applications of ceragenins as stand-alone antimicrobial agents and in medical device coatings.

7.2. ANTIMICROBIAL PEPTIDES: DEFINITION, SOURCE AND HISTORY

AMPs are evolutionarily conserved molecules that exist in organisms ranging from prokaryotes to humans as a first line of defense [1,2]. They are naturally found in the skin, airways, gastrointestinal tract, and urinary and reproductive tracts with the highest concentrations of AMPs being found in the tissues that are exposed to pathogens [3,4]. The first human AMP described, lysozyme, was discovered in 1922 by Alexander Fleming from his own nasal mucus. Further investigation demonstrated that lysozyme targets bacteria by hydrolyzing the
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linkage of peptidoglycan of the cell wall in Gram-positive bacteria [5-8]. However, antimicrobial use of lysozyme was overshadowed by the discovery of penicillin in 1928, also by Fleming [9]. The emergence of drug-resistant pathogens in the 1960s brought the potential of therapeu tic use of AMPs again to the attention of scientists. Pioneering work in 1956 resulted in discovery of defensins, the first animal-originated AMPs, isolated from rabbit leukocytes [10]. This was followed by the report of lactoferrin from milk in the 1960s [11]. Cecropin, first isolated from the Cecropia silk moth *Hyalophora cecropia* in 1981, was the first helical AMP reported [12,13]. Zasloff and colleagues isolated magainins from the skin of the African clawed frog *Xenopus laevis* in 1987 and found that it displayed broad spectrum antimicrobial activity [14]. It is also worth noting that prokaryotes produce and release AMPs to fight other microorganisms in their environment; however, these AMPs mostly have cyclic or branched structures. These AMPs are often synthesized by a non-ribosomal peptide synthase through a specialized metabolic pathway, unlike eukaryotic AMPs which are synthesized by the ribosome. To date, over 250 of these bacteriocins have been identified from bacteria [9]. Of particular interest are the commercial antibiotics polymyxin B (cationic) and vancomycin (non-cationic), which are isolated from *Bacillus polymyxa* and *Amycolatopsis orientalis*, respectively. Polymyxin B displays potent antibacterial activity against Gram-negative bacteria and vancomycin against Gram-positive bacteria [15,16].

7.3. STRUCTURE AND MECHANISM OF ACTION OF AMPS

AMPs mainly fall into three classes: α-helical, β-sheet, and extended, depending on their secondary structures [17,18]. Magainins and the human cathelicidin LL37 (hereafter LL-37) are prominent examples of AMPs with substantial α-helical structure [19,20]. β-sheet AMPs include defensins and feature disulfide bond that stabilize their structure [21]. AMPs derived predominantly from one type of amino acid are categorized as extended AMPs and include human histatins, which are rich in histidine, and bovine indolicidin, which is rich in tryptophan and arginine [22].

Nearly all AMPs are amphipathic, meaning they have both cationic and hydrophobic character. However, unlike typical surfactants, with polar head groups and hydrophobic tails, AMPs display polar, cationic functionality on one face of the molecule with hydrophobic groups juxtaposed on the opposite face [23]. Defensins and cathelicidins constitute the major families of membrane-active AMPs in vertebrates [24]. And with these AMPs, an initial, selective electrostatic interaction between the positive charges on one face of the AMPs and negative charges of membranes occurs. Due to their facial amphiphilicity, the hydrophobic domains of AMPs cannot stably insert into intact membranes,
and at sufficient local concentrations, AMPs cause membrane perturbation leading to a loss of polarization, resulting in cell death. Membrane composition allowing selective association of AMPs with microbial membranes, plays a key role in cell selectivity [25]. For example, magainins show higher affinity for anionic phospholipids, which are highly represented in bacterial membranes. Cell membranes of higher organisms, however, feature neutral phospholipids and cholesterol, which diminish AMP interactions [26,27]. For this reason, much higher concentrations of AMPs are required to kill cells from higher organisms as compared to microbial cells. Targeting of the cell membranes by AMPs gives rise to their breadth of spectrum of activity: bactericidal activity against Gram-negative and positive bacteria, antifungal activity and even activity against lipid-enveloped viruses. This breadth of activity may offer advantages over antibiotics that target specific cellular processes, such as DNA and protein synthesis, leading to narrow spectra of activity. Membrane-targeting properties of AMPs contribute to the rapid antimicrobial activity, with some AMPs able to kill microorganisms with only seconds of exposure [13,28].

In higher eukaryotic organisms, AMPs display immunomodulatory activities [29], including anti-inflammatory properties through the sequestration of bacterial endotoxins such as lipopolysaccharide (LPS). In addition, multiple studies support the idea that human AMPs improve wound healing via promotion of cell migration into wound beds, angiogenesis, and neovascularization [30]. AMPs are generally constitutively expressed in healthy epithelium, and their production is upregulated in response to injury or infection to moderate microbial proliferation and signal host cells to activate secondary immunomodulatory roles. For example, a study with a human skin wound healing model, LL-37 was expressed acutely post-injury, whereas its concentration was reduced in chronic ulcer epithelium where re-epithelization is impaired, suggesting that LL-37 plays a prominent role in wound closure [31].

Despite the broad-spectrum antimicrobial activities of AMPs and their wound-healing and immunomodulatory properties, there are factors that limit their clinical applications. These include their susceptibility to proteases released by bacteria, the high cost of producing them on a large scale, decreased activity when immobilized, and folding problems in the production of some large AMPs [13].

We developed ceragenins as non-peptide mimics of AMPs [32]. They are synthesized from cholic acid, a common bile acid, in few synthetic steps (Figure 1) [33-36]. Due to the fact that they are not peptide-based, they are not substrates for ubiquitous proteases. Preparation and purification of ceragenins on a large scale is relatively straightforward, they are stable under physiological conditions and even long-term storage in solution does not reduce their antibiotic activities [37,38]. As ceragenins follow the amphiphilic
structure of AMPs they display a similar spectrum of activity against bacterial, fungi and lipid-enveloped viruses [39].

Figure 1. Structures of selected ceragenins CSA-13, CSA-131, CSA-142, CSA-44 and CSA-144

7.4. DESIGN OF CERAGENINS

Preliminary design of the ceragenins (examples in Figure 1) was based on the presumed lipid A binding domain of polymyxin B. The primary amines from diaminobutyric acid groups in polymyxin B are oriented on one face of the molecule, and to mimic this arrangement, three primary amines were tethered to cholic acid to generate similar amine spacing in ceragenins. Tether lengths were incrementally varied to determine the optimal spacing between the steroid backbone and the amine groups [40-43]. These initial attempts to mimic the structure of polymyxin B resulted in preparation of ceragenins with activity against *Eschericia coli* [minimal inhibitory concentration (MIC) range 2.0–46 µg mL$^{-1}$]. A truncated form of polymyxin B, polymyxin B nonapeptide, permeabilizes the outer membranes of Gram-negative bacteria, sensitizing them to hydrophobic antibiotics. With the initial series of ceragenins, this type of sensitization was observed with erythromycin against *E. coli*; concentrations of CSA-8 as low as 1 µg mL$^{-1}$ lowered the MIC of erythromycin from 70 to 1 µg mL$^{-1}$ [40,44]. Further structure-activity studies demonstrated that the chain extending from the D ring of the bile acid played a central role in controlling direct antibacterial activity vs. sensitizing activity of Gram-negative bacteria. With lipophilic groups attached to this chain, the resulting ceragenins displayed broad-spectrum antibacterial activity (Gram-negative and -positive). For example, CSA-13 gave an MIC of 3.0 µg mL$^{-1}$ against *E. coli* (Gram-negative) and 0.40 µg mL$^{-1}$ against *Staphylococcus aureus* (Gram-positive). Ceragenins lacking lipophilic groups on the D-ring retained antibacterial activity against Gram-positive bacteria but lost substantial activity against Gram-negative bacteria. For example, CSA-8, without an extensive lipid chain, gave an MIC of
Ceragenins as non-peptide mimics of endogenous antimicrobial peptides

36 µg mL\(^{-1}\) against *E. coli*, and an MIC of 2.0 µg mL\(^{-1}\) against *S. aureus*. Nevertheless, ceragenins lacking lipophilic groups extending from the D ring retained the ability to sensitize Gram-negative bacteria to hydrophobic antibiotics [45].

From these results, we postulated that lipid chains extending from the D ring enabled ceragenins to traverse the outer membrane and exert antibacterial activity through interactions with the cytoplasmic membrane. Gram-positive bacteria lack an outer membrane, and they are susceptible to ceragenins with and without a lipid chain. Ceragenins lacking a lipid chain extending from the D ring remain on the outer surface of the outer membrane, and association of these ceragenins with the lipid A component of the membrane causes perturbations in the membrane that allow hydrophobic antibiotics to traverse the membrane [35,40,44].

Further structure activity studies involved replacement of the ether bonds tethering the amines to the bile acid with amide and ester groups. To use amide groups in the tethers, it was necessary to synthesize a tri-amino version of cholic acid [46]. These amines were functionalized with amino acids, generating amides. The resulting ceragenins displayed antibacterial activity, but less than that of the ceragenins with ether bonds for the tethers. By differentially protecting the three amines in the tri-amino version of cholic acid, it was possible to sequentially incorporate different amino acids at each amine. Although this method of preparing ceragenins resulted in the production of large numbers of compounds, none were as active as ether-based compounds. The ester-based compounds (*e.g.*, CSA-44) were prepared from cholic acid, and some of the resulting compounds showed activity that rivaled that of the ether-based compounds. A feature of the ester-based compounds is that the ester groups spontaneously hydrolyzed in water (half-life of 37 days at pH 7). Hydrolysis of all of the esters gave cholic acid, an amino acid and a waxy alcohol [47-49].

7.5. MECHANISM OF ACTION OF CERAGENINS

A primary mode of antimicrobial activity of AMPs involves selective association with microbial membranes, and this association is mediated by ion pairing of positively charged AMPs with negatively charged microbial membranes [50]. Similarly, ceragenins selectively associate with negatively charged components of bacterial cell membranes, and ceragenins have been shown to sequester bacterial endotoxins (*e.g.*, LPS and lipoteichoic acid), which make up membranes of Gram-negative and positive bacteria [51,52]. This affinity for bacterial membrane components translates into cell selectivity; in studies with intact cells, ceragenins were shown to selectively associate with
prokaryotes over mammalian cells, and this selective affinity is likely due to the higher net negative charge present on prokaryotic membranes [25].

As a consequence of the interactions of AMPs with the outer membranes of Gram-negative bacteria, blebs form on the surface [53-55], and similar morphological changes occur with ceragenin-treated, Gram-negative bacteria [44]. This observation, along with measurement of affinity of ceragenins for the lipid A portion of lysophosphatidic acid (LPA) and the cell selectivity described above, attest to the interactions between ceragenins and the outer membranes of Gram-negative bacteria. However, perturbation of the outer membrane is insufficient for cell death, and the ultimate target must be the cytoplasmic membrane [56,57]. As described above, a lipid chain extending from C24 is required for ceragenins to traverse the outer membrane of Gram-negative bacteria and gain access to the cytoplasmic membrane. A ML-35p mutant strain of \textit{E. coli} was used to show that ceragenins containing such a lipid chain traverse the outer membranes of Gram-negative bacteria and to gain access to the cytoplasmic membrane [36]. Association of ceragenins with the cytoplasmic membranes of bacteria, in sufficient concentrations, leads to formation of transient membrane defects, membrane depolarization and cell death. This mechanism of action is best described by the carpet model proposed for the activity of AMPs with bacteria [25]. Although antiviral and antifungal mechanisms of action of ceragenins are not completely understood, electron micrographs of ceragenin-treated vaccinia virus exhibited considerable morphological changes in the viral lipid envelope, and ceragenin-treated fungal cells displayed changes in cell shape, similar to that observed with AMPs [58].
7.6. ANTIMICROBIAL SPECTRUM OF ACTIVITY OF CERAGENINS

7.6.1. Antibacterial activity

The antimicrobial activities of ceragenins have been studied more with bacteria than with other organisms (e.g., fungi and viruses). While they display activity against both Gram-positive and Gram-negative bacteria, ceragenins tend to be active at lower concentrations against Gram-positive bacteria, possibly due to the permeability barrier of the outer membranes of Gram-negative bacteria. Ceragenins have shown activity against a wide array of clinical isolates and drug-resistant bacteria, supporting their potential for clinical use as the threat of antibiotic-resistant bacteria grows [45, 59]. To highlight the broad-spectrum antibacterial activities of ceragenins, their abilities to eliminate drug-organisms are highlighted below.

Susceptibility experiments of CSA-13 with four clinical isolates of vancomycin-resistant *S. aureus* and 50 clinical isolates of glycopeptide-intermediate and heterogeneous glycopeptide-intermediate *S. aureus* showed that the MIC<sub>50</sub>, MIC<sub>90</sub> and MBC of all isolates was 1 µg mL<sup>−1</sup> [60]. These results demonstrate that there is no cross reactivity between glycopeptides and ceragenins. A more pressing concern may be with multi-drug resistant Gram-negative pathogens, and in a study with 60 carbapenem-resistant *Acinetobacter baumannii* strains isolated from blood specimens of bacteremia patients, CSA-13 gave MIC<sub>50</sub> and MIC<sub>90</sub> of 2 and 8 µg mL<sup>−1</sup>, respectively. Similar results were observed with 50 strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients [61].

Resistance to antibiotics of last resort, such as colistin, is of particular concern [62]. Colistin, AMPs, and ceragenins share common structural features (e.g., multiple cationic amines and hydrophobic character), and this led to the concern that colistin-resistance might also confer resistance to AMPs and ceragenins. A study of selected ceragenins (CSA-13, CSA-44, CSA-131, CSA-138 and CSA-142) and AMPs (LL-37, megainin 1 and cecropin A) revealed that both ceragenins and AMPs are active against highly colistin-resistant *Klebsiella pneumoniae* isolates. Furthermore only minor differences were observed in the kinetics of the bactericidal activity of ceragenins among the colistin-resistant and colistin-susceptible strains, indicating that colistin-resistance does not remarkably influence the susceptibility of these pathogens to ceragenins [63].

In a separate study, assessment of four ceragenins, CSA-138, CSA-13, CSA-131 and CSA-44, against clinical isolates of colistin-resistant and susceptible *P. aeruginosa* (MIC 0.5–1 µg mL<sup>−1</sup>) and *A. baumannii* (MIC 2–8 µg mL<sup>−1</sup>) showed that MICs among susceptible and resistant strains are identical or comparable [63].

Ceragenins have also shown activity against cariogenic and periodontopathic bacteria [37]. Susceptibilities of broad-spectrum pathogens associated with oral and upper respiratory tract infections including *Streptococcus mutans* (the
leading etiological agent of dental caries) and *S. aureus* (which often colonizes the nasopharynx) were determined with ceragenins CSA-13, CSA-90 and CSA-92 and compared to LL-37. As shown in Table 1, all tested ceragenins revealed significantly potent antibacterial activity when compared to LL-37 [64]. Interestingly, *Lactobacillus casei*, considered a beneficial member of the gut microflora, was much less susceptible to ceragenins and LL-37 than other organisms, indicating a possible adaptation of this strain to become tolerant of LL-37 and probably ceragenins.

**Table 1.** MIC [MBC] µg mL$^{-1}$ of LL-37 and ceragenins against tested strains associated with oral infections

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<tbody>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 29213</td>
<td>14 [28]</td>
<td>0.7 [1.4]</td>
<td>0.7 [2.8]</td>
<td>0.75 [0.75]</td>
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<tr>
<td><em>Streptococcus salivarius</em> ATCC 13419</td>
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<td>0.7 [1.4]</td>
<td>0.7 [1.4]</td>
<td>1.5 [3.0]</td>
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<td>1.6 [1.4]</td>
<td>1.5 [3.0]</td>
</tr>
<tr>
<td><em>Streptococcus mutants</em> ATCC 35668</td>
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<td>0.7 [1.4]</td>
<td>0.75 [1.5]</td>
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<tr>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td>28 [56]</td>
<td>2.8 [2.8]</td>
<td>1.4 [2.8]</td>
<td>8.0 [3.0]</td>
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<tr>
<td><em>Moraxella catarrhalis</em> ATCC 23246</td>
<td>28 [28]</td>
<td>1.4 [1.4]</td>
<td>0.7 [1.4]</td>
<td>0.35 [1.5]</td>
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<tr>
<td><em>Lactobacillus casei</em> ATCC 393</td>
<td>224 [224]</td>
<td>22.4 [44.8]</td>
<td>44.8 [44.8]</td>
<td>46.8 [46.8]</td>
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The permeability barrier of the outer membranes of Gram-negative bacteria and the action of efflux pumps in these membranes contribute to resistance profiles of bacteria [65]. Association of ceragenins with the outer membranes decreases the permeability barrier resulting in synergy between ceragenins and other antibiotics [66]. For example, synergistic activities of CSA-13 were reported with colistin, tobramycin, and ciprofloxacin against *P. aeruginosa* strains isolated from cystic fibrosis patients. The synergistic effect of CSA-13 was stronger with colistin (54% of tested strains) compared to tobramycin (25% of tested strains) [67]. Further investigation showed that CSA-13 also displays synergistic activity with cefepime or ciprofloxacin against clinical
isolates of *P. aeruginosa*, including multidrug-resistant *P. aeruginosa* [68]. In related studies, the synergistic effects of ceragenins with AMPs were studied; combinations of CSA-13 with selected AMPs LL-37, lysozyme, lactoferin, or secretory phospholipase A were evaluated against bacteria causing topical infections. As expected, CSA-13 exhibited more potent antibacterial activity in the presence of all tested AMPs [69].

### 7.6.2. Antifungal activity

The continued emergence of drug-resistant fungal pathogens has highlighted the urgent need for novel antifungal agents [70]. AMPs display antifungal activity [13] and this observation led to the question of whether ceragenins, as mimics of AMPs, would also display antifungal activity. Fungicidal activity of ceragenins CSA-13, CSA-131 and CSA-192 was evaluated against four fluconazole–resistant Candida strains [71]. Interestingly, these ceragenins showed higher antifungal activity against these strains than LL-37 and omiganan, a synthetic AMP designed for antifungal activity. In addition to *Candida* spp., CSA131 and CSA-192 were also found to be active against a broad array of fungal strains including *Cryptococcus, Aspergillus, Scedosporium, Rhizopus* and *Blastomyces*.

Recently, the global emergence of invasive infections caused by drug-resistant *Candida auris* has become a serious threat to public health. A high percentage of clinical isolates of *C. auris* are resistant to fluconazole and at least one of the other major classes of antifungals (polyenes and echinocandins) [72,73]. Already shown to be active against *Candida albicans*, lead ceragenins were selected for testing against *C. auris* [74]. The Center for Disease Control and Prevention determined the susceptibility of 100 clinical isolates of *C. auris* to CSA-131. They found that the MIC$_{50}$ and the MIC$_{90}$ were 1 µg mL$^{-1}$, and no loss of activity of CSA-131 was found among fluconazole-resistant or echinocandin-resistant isolates. Further studies with a smaller collection of *C. auris* isolates were performed with ceragenins CSA-13, CSA-44, CSA-131, and CSA-144, along with caspofungin, amphotericin B and fluconazole, representatives of the three major classes of antifungal agents in clinical use. The selected ceragenins showed MICs comparable to those of caspofungin and amphotericin B; however, as expected fluconazole was weakly active against the *C. auris* strains (Table 2). Notably, minimum fungicidal concentrations (MFCs) of the ceragenins were significantly lower than those of caspofungin and amphotericin B with most of the clinical isolates.
Table 2. MIC [MFC] µg mL\(^{-1}\) of ten clinical isolates of *C. auris* to selected ceragenins and three major classes of antifungal agents

<table>
<thead>
<tr>
<th>Strains</th>
<th>CSA-44</th>
<th>CSA-131</th>
<th>CSA-142</th>
<th>CSA-144</th>
<th>CPF</th>
<th>AMB</th>
<th>FLZ</th>
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<tr>
<td><em>C. auris</em></td>
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<td>16</td>
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<td>[2.0]</td>
<td>[8.0]</td>
<td>[32]</td>
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<td><em>C. auris</em></td>
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CPF: caspofungin, AMB: amphotericin B, FLZ: fluconazole. nm: not measured

The primary antifungal mechanism of action of AMPs is proposed to involve interactions and potentially disruption of the fungal membrane. Apoptosis as a result of increased reactive oxygen species (ROS) and decreased mitochondrial function have been also observed [75-78]. Although the antifungal mechanism of ceragenins is not still completely understood, atomic force and scanning electron microscopy of candida cells treated with ceragenins shows significant changes in the surface cell morphology compared to untreated cells (Figure 2), suggesting they act *via* interactions with fungal membranes and similarly to AMPs. In a recent study, LL-37 or CSA-13 immobilized on magnetic nanoparticles were to shown to increase ROS generation in fungal cells, suggesting an additional pathway of antifungal activity with *Candida* spp. (Figure 3) [79]. Nevertheless, additional information is still required to fully understand how ceragenins target and kill fungal cells.
Ceragenins as non-peptide mimics of endogenous antimicrobial peptides

Figure 2. Scanning electron microscope (SEM) images of untreated *C. auris* CDC390 (A), treated with CSA-131 25 mg L$^{-1}$ (B) and 50 mg L$^{-1}$ (C)

Figure 3. Proposed mechanism of positively charged coated magnetic nanoparticles with LL-37 or CSA-13 against fungal cells membrane and contribution of LL-37/CSA-13 to generation of ROS

1. Interaction of MNP@LL-37/CSA-13 to the cell membrane
2. Disruption of cell membrane
3. Internalization of MNP@LL-37/CSA-13 and ROS generation
4. Cell death
7.6.3. Antibiofilm activity

Bacteria and fungi naturally exist in planktonic and biofilm forms. Planktonic cells are single, free-growing microorganisms, while biofilms form from groups of cells that aggregate through a protective extracellular polymeric matrix [80]. Biofilms are now thought to be involved in most infections, including chronic wounds, medical device-related infections, and cystic fibrosis-associated pneumonia. Eradication of biofilms can be a particularly daunting task due to the protective environment provided by the extracellular matrix and because cells within the biofilm are slow-growing and have low metabolic activity and therefore do not respond to antibiotics that target and inhibit cells that are rapidly growing and reproducing [81]. These factors lead to a 100–1,000-fold increase in the ability of the bacteria or fungi to resist antibiotic treatment, compared to planktonic cells [13]. For example, β-lactam antibiotics inhibit the synthesis of the peptidoglycan layer of the bacterial cell wall and while their effect is considered bactericidal, it is most effective against cells that are growing and preparing to divide, as they require more peptidoglycan [82]. Consequently, β-lactams lose activity as bacteria transition from planktonic to sessile forms found in biofilms. Ceragenins and AMPs, on the other hand, target the bacterial membrane, and are able to kill bacterial and fungal cells regardless of whether they are rapidly growing, dividing, or sessile [83]. In addition, the relatively small size of ceragenins allows them to penetrate the extracellular matrix of biofilms and gain access to the cells within [38]. A comparative study of ceragenins with ciprofloxacin indicated that a much lower concentration of CSA-13 was required to eradicate an established biofilm consisting of a meticillin-resistant strain of *S. aureus* in comparison with ciprofloxacin. While treatment with CSA-13 caused a complete eradication of biofilm at a concentration between 16 and 32 µg mL⁻¹, treatment with ciprofloxacin resulted in an insignificant reduction of biofilm even at a concentration of 64 µg mL⁻¹ [84]. In a similar study, it was observed that CSA-13 inhibits biofilm formation of both mucoid and nonmucoid phenotypes of *P. aeruginosa* at 1 µg mL⁻¹ [85]. It was also noted that the rhlAB operon, which is controlled by quorum sensing and regulates rhamnolipid synthesis, was not an intracellular target of CSA-13 involved in the inhibition of biofilm formation. Further study with confocal laser scanning images of biofilms formed by *P. aeruginosa* revealed that bactericidal penetration of ceragenins into the biofilm matrix occurs within only 30 minutes of exposure of the ceragenin to the established biofilm [86].

In a recent study, the ability of clinical isolates of *C. auris* and *C. albicans* to form biofilms was quantified by measuring metabolic activity through use of XTT reagent in a colorimetric assay [74]. Under identical conditions, *C. auris* showed less metabolic activity than *C. albicans*, indicating a lower ability of *C. auris* to form biofilm compared to *C. albicans*. The concentrations of ceragenins necessary to reduce biofilms by 50 and 80% were then determined. Ceragenins revealed similar or superior antibiofilm activity against sessile
organisms in established biofilms when compared to amphotericin B, caspofungin, and fluconazole.

7.6.4. Sporicidal activity
Some genera of bacteria such as *Bacillus* and *Clostridium* undergo a process of sporulation in response to unfavorable environmental conditions including depletion of nutrients [87]. Sporulation enables bacteria to form inert, dormant spores, and this process involves formation of a multilayered structure different than vegetative cells [88]. Given the membrane-permeabilizing properties of ceragenins, activity of CSA-13 against the vegetative and spore forms of *B. subtilis* was assessed [89]. Treatment with CSA-13 significantly diminished the viability of vegetative cells and inhibited spore germination. Moreover, the surface electrical features of spores and vegetative cells measured through zeta potential provided evidence that the surfaces of spores exhibit a larger negative charge than vegetative cells. It was postulated that this charge density was the cause of the high affinity of CSA-13 spore surfaces. Raman spectroscopy analysis further illustrated that ceragenin-treated spores release more calcium dipicolinic acid than untreated cells, implying that there is an increased permeability in the barriers of these spores, which increases their susceptibility to ceragenins.

7.6.5. Antiviral activity
AMPs exhibit antiviral activity through several mechanisms. First, by targeting the viral envelopes of lipid-enveloped viruses and disrupting membrane integrity, AMPs cause viruses to lose the ability to infect the host cells. Second, AMPs block viral receptors on the cell surface and prevent viruses from binding to host cells [13]. This can be seen in the interaction between θ defensins and viral glycoproteins of the Herpes simplex virus [90]. The antiviral activity of LL-37 against vaccinia virus has been established, and subsequently the antiviral activity of CSA-13 was studied against the same virus, which is a large double-stranded DNA virus that can infect a wide variety of mammalian cells as well as invertebrate cells [58]. CSA-13 showed potent antiviral activity through targeting the viral envelope. Additionally, topical application of CSA-13 in a murine model of vaccinia infection showed a substantial reduction in the number of satellite lesions that formed and in viral replication in the epidermis of infected mice.

7.6.6. Anti-parasite activity
The actions of a few AMPs have been studied with parasites, and anti-parasitic activities have been attributed to direct interactions with cell membranes. Magainins were reported as the first AMPs with anti-parasitic properties, displaying activity against *Paramecium caudatum* [14]. Cathelicidin is another example of an anti-parasite AMP, with activity against *Caernohabditis elegans*
through pore formation on the cell membrane [91]. CSA-13 was also tested for anti-parasitic activity and showed an LD\(_{50}\) of ca. 9 and 5 μM with *Trypanosoma cruzi* and *Leishmania major*, respectively [92]. *Acanthamoeba castellanii* is a causative agent of corneal infections known as *acanthamoeba keratitis*. The parasite has two phases of life cycle, including cysts (the most resistant form) and trophozoites (the replicating stage) [93]. An *in vitro* evaluation of CSA-13 at concentrations of 25, 50, 75, and 100 mg mL\(^{-1}\) against both cyst and trophozoite forms of *A. castellanii* showed that CSA-13 inhibited trophozoite growth in a dose- and time-dependent manner. For example, within one hour of exposure to 100 mg mL\(^{-1}\) CSA-13, no viable trophozoites were detected [94]. Further study was performed using ceragenins CSA-13, CSA-44, CSA-131, and CSA-138 against metronidazole-susceptible and metronidazole-resistant strains of *Trichomonas vaginalis*, a parasitic protozoan transmitted via sexual intercourse and hosted only in humans. Overall, all tested ceragenins killed the tested *T. vaginalis* with a similar activity against metronidazole-susceptible and resistant strains, and CSA-13 was the most effective ceragenin in this study [95].

### 7.7. MICROBIAL RESISTANCE TO AMPS AND CERAGENINS

AMPs have existed for eons and have played a central role in nearly all organisms in controlling the growth of pathogens. Even most common pathogens have been exposed to AMPs for extended periods, most remain susceptible to AMPs, while pathogens have developed high levels of resistance to most other clinically-used antibiotics [96]. The cause of the enduring activity of AMPs may be due to a variety of factors: First, AMPs have co-evolved with the pathogens they are fighting, and through natural selection AMPs with optimized properties are selected for as they increase the fitness of their host. Second, while some AMPs are constitutively expressed, some are only released or have their expression substantially up-regulated with infection or inflammation. This non-constitutive and localized expression may also hinder a pathogen's ability to form resistance as it never is allowed to adapt to an environment that contains sub-therapeutic levels of AMP [97]. Finally, AMPs target the membranes of microorganisms rather than specific enzymatic pathways. Consequently, resistance to AMPs requires modification to a gross structural component of microorganisms, a process that may come with a metabolic cost or substantial changes in interactions of the microorganisms with their environment [98]. However, bacteria are not totally defenseless against AMPs [13]. Some bacterial pathogens, for example *S. mutans*, produce and release metalo-proteases, and it has been hypothesized that these are designed to destroy AMPs. Prolonged exposure of Gram-negative bacteria to AMPs results in modifications to the lipid A portion of LPS. For example, in
Salmonella typhimurium this modification is caused by two-component signal transduction systems including PhoP-PhoQ and PmrA-PmrB [99]. The presence of positive charges on AMPs activates PhoQ, which is a membrane-associated protein kinase, and activated PhoQ phosphorylates PhoP, which leads to upregulation of a host of proteins including PagP leading to the addition of palmitate to membrane-associated lipid A. This change increases the hydrophobic character of lipid A and membrane stiffness. The same signal transduction system also leads to further modification of lipid A through formation of phosphate esters with ethanolamine and L-4-amino arabinose [100-102]. These changes decrease the net negative charge of the membrane and further decreases the affinity of AMPs to the bacterial membrane. These defenses, however, are metabolically costly to bacteria, and absent AMPs or other threats, bacteria shut down these systems and exist in AMP-susceptible forms. It is also interesting to note that although resistance to AMPs has been observed in vitro, no evidence of resistance in vivo has been observed to this point [30].

To investigate the potential for bacteria to generate resistance to ceragenins, selected Gram-negative and Gram-positive bacteria were serially passaged with varied concentrations of lead ceragenins CSA-13 and CSA-131 [63,103]. Comparator antibiotics were ciprofloxacin and vancomycin with Gram-positive bacteria and ciprofloxacin and colistin with Gram-negative bacteria. With both Gram-positive and -negative bacteria, MICs with ciprofloxacin increased to high levels (>50 μg mL⁻¹) with relatively few passages of 24 h. The MIC of vancomycin increased ca. 10-fold with S. aureus over 30 passages of 24 h, while only a small change of MIC (less than two-fold) occurred over the same number of passages with CSA-13 [103]. With Gram-negative bacteria, the MIC of colistin increased to >100 μg mL⁻¹ within 20 passages of 24 h, and over 30 passages of 24 h, the MIC of CSA-13 increased to 20-30 μg mL⁻¹. In contrast, the MIC of CSA-131 from 1–2 μg mL⁻¹ to 2–8 μg mL⁻¹ over 30 passages of 24 h with P. aeruginosa, and A. baumannii and Klebsiella pneumoniae (Figure 4) [63].
To understand Gram-negative bacterial responses to serial passaging with colistin or CSA-131, lipid A was isolated from the membranes of serially-passaged strains and analyzed via mass spectroscopy [63]. As is the case with AMP-resistance, modification of lipid A through the addition of 4-
aminorabinose, ethanolamine, and fatty acids was observed with strains exposed to either colistin or CSA-131, likely as a result of the activation of two-component systems such as PhoP/PhoQ and PmrA/PmrB. However, as seen previously, the highly colistin resistant strain was fully susceptible to CSA-131, suggesting that mechanisms other than lipid A modification are likely involved in colistin resistance. These results offer evidence that the ceragenins are less likely to engender resistance than other antibiotics.

The antimicrobial activities of AMPs and ceragenins may be influenced not only by modifications made by bacteria but also by production of specific molecules produced by the host. Because mucins, DNA and F-actin have been shown to deactivate AMPs, the question arose of how these compounds would impact the activities of ceragenins. Comparative studies showed that these molecules had much less impact on the antibacterial activities of ceragenin CSA-13 than AMPs [52,104]. This observation is particularly important for potential applications of ceragenins in treating infections associated with cystic fibrosis. Mucins, DNA and F-actin are produced at relatively high concentrations in the lungs of those with cystic fibrosis. The smaller size of CSA-13 and lower positive charge, relative to endogenous AMPs, were proposed to be why the antibacterial activity of CSA-13 is less compromised by mucins, DNA and F-actin than AMPs. In a following study, the effects of selected depolymerizing factors (plasma gelsolin, DNase 1, and poly-aspartate) on polyelectrolyte networks of F-actin and DNA in purulent body fluids were assessed in order to determine if they would improve the bactericidal activities of CSA-13, LL-37, tobramycin, colistin and polymyxin [105]. The depolymerizing compounds were found to enhance the activities of the tested antimicrobials, particularly CSA-13 and LL-37. Stronger antibacterial activity was observed with combination of DNase 1 and poly-aspartate as compared to the individual depolymerizing compounds [105]. These results provide strong evidence that because ceragenins possess potent antibacterial activity that is even sustained in the presence of complex anionic compounds, they offer excellent potential in treating chronic lung infections in cystic fibrosis patients, where the accumulation of polyanions creates major treatment obstacles.
7.8. EFFECT OF POLOXAMER MICELLES ON THE ANTIMICROBIAL ACTIVITIES AND CYTOTOXICITY OF CERAGENINS

Clinical use of AMPs and ceragenins requires selective toxicity for microorganisms over host cells. One measure of cytotoxicity used to evaluate membrane-active compounds is measurement of concentrations of investigational antimicrobials that cause hemolytic activity. One such study showed that CSA-13 is not hemolytic at concentrations required for bactericidal activity and hemolysis was first observed at concentrations 10 times the bactericidal concentration [52]. Further evaluation of hemoglobin release from human red blood cells showed that CSA-13 at a concentration of 10 mg mL\(^{-1}\) causes lysis in less than 10% of erythrocytes while total hemolysis occurs at a concentration of 50 mg mL\(^{-1}\) [106]. These concentrations are higher than the concentrations required for bactericidal activity of ceragenins. In studies with HaCat cells, the toxicity profile of CSA-13 was similar to that of LL-37, without any toxicity at bactericidal concentrations [64].

Cytotoxicity was explored in a comparative study of ceragenins with bacterial cells and human umbilical vein endothelial cells. It was found that CSA-13 permeabilizes the plasma membrane of these eukaryotic cells in addition to the bacteria cells [107]. In an attempt to attenuate this effect of CSA-13 on eukaryotic cells, it was combined with the poloxamer Pluronic® F-127, a nonionic surfactant comprised of polyoxyethylene–polyoxypropylene copolymers (hereafter referred to as pluronic). The low toxicity of this poloxamer, great solubilizing capacity, and unique thermoreversible and drug releasing properties contribute to its applications in drug delivery as a pharmaceutical vehicle [108]. This poloxamer forms micelles that associate with hydrophobic and amphiphilic compounds. Formulation of ceragenins with pluronic resulted in well-defined micelles with lower toxicity toward eukaryotic cells, while antibacterial activity was not substantially impaired [69,109]. Studies included co-formulation of ceragenins and pluronic and showed that association of ceragenins in poloxamer micelles allowed them to maintain their antibacterial and antifungal activities against planktonic organisms as well as established biofilms.

An additional means of determining possible cytotoxicity of ceragenins involves use of ciliated explants from the trachea or lung. Epithelial cells in these tissues produce cilia, which beat to move particles from the trachea and lung to the throat. Thus cilia beating can act as a surrogate for measurement of even minor insults to epithelial cells [110-112]. Using tissue explants, high concentrations of ceragenin CSA-131 were shown to negatively impact cilia beating. However, formulation of CSA-131 with pluronic resulted in complete retention of cilia beating, indicating that the underlying epithelial cells were unaffected even by relatively high concentrations of CSA-131 (100 µg mL\(^{-1}\)). Furthermore, SEM images (Figure 5) of ciliated trachea showed that CSA-131
formulated with pluronic left cilia intact, while CSA-131 at 100 µg mL\(^{-1}\) resulted in loss of cilia. The antifungal activity of CSA-131 was entirely sustained in the infected trachea and lung. Therefore, CSA-131 formulated in micelles may act as an appropriate candidate for the treatment of polymicrobial and biofilm-related infections in lung and trachea [113].

![Figure 5](image)

**Figure 5.** SEM images of ciliated surface of a porcine trachea explants. Untreated (A), treated with CSA-131 at 100 µg mL\(^{-1}\) (B), treated with CSA-131 at 100 µg mL\(^{-1}\) with 4 % pluronic (C). Exposed goblet cells are shown with white squares in the image from treatment with CSA-131 without pluronic.

### 7.9. NANOPARTICLES AS CARRIERS OF CERAGENINS

Metal-based nanoparticles provide a unique platform for the presentation of ceragenins in a pre-aggregated form. In addition, the distribution of magnetic nanoparticles can be controlled in tissues and silver-based nanoparticles display inherent antimicrobial activity. Multiple methods have proven effective in attaching ceragenins to nanoparticles. One method uses CSA-124, a ceragenin formulated with a thiol group appendage. The thiol group can coordinate with thiophilic metals, such as silver, producing a monolayer on the surface of the nanoparticles [114]. Another method requires the generation of an aldehyde-containing surface on the nanoparticles. Ceragenins contain multiple amine groups, and these groups reversibly form Schiff bases with the aldehydes. On a surface with high aldehyde density, nanoparticles can then aggregate ceragenins in high concentrations [115].

Hoppens *et al.* [114] coated silver nanoparticles with CSA-124 and found that they were five times more bactericidal than silver alone. These nanoparticles displayed MICs of ca. 12 and 24 ppm against *S. aureus* and *E. coli*, respectively. Niemirowicz *et al.* [115] showed that magnetic nanoparticles coated with ceragenins retained their bactericidal activity. This association also increased the biocompatibility of ceragenins. Further testing also showed that even
without direct conjugation of ceragenin to the nanoparticle, synergistic activity between nanoparticles and ceragenins occurred [116].

Durnás et al. [117] also evaluated magnetic nanoparticles for their activity against selected anaerobic bacteria often implicated in human disease. In vitro models testing CSA-13 and CSA-131 attached to nanoparticles showed comparable or stronger bactericidal activity against all of the tested strains, which included *Bacteroides fragilis*, *Propionibacterium acnes*, and a clinical isolate of *Clostridium difficile* (Table 3). Nanoparticles including CSA-131 also showed enhanced ability in preventing biofilm formation of *Bacteroides fragilis* and *Propionibacterium acnes*. Neimirowicz et al. [79] further studied the candidacidal activity of magnetic nanoparticles coated with CSA-13 and CSA-131. Their experiments confirmed that ceragenins attached to nanoparticles retained their highly potent fungicidal activity against multiple laboratory and clinical species of *C. albicans*, *C. glabrata*, and *C. tropicalis*.

**Table 3.** MIC [MBC] µg mL⁻¹ of LL-37, CSA-13 and CSA-131 functionalized on nanoparticles against tested anaerobic strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>LL-37</th>
<th>MNP @ LL-37</th>
<th>CSA-13</th>
<th>MNP@ CSA-13</th>
<th>CSA-131</th>
<th>MNP@ CSA-131</th>
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<tr>
<td><em>Bacteroides fragilis</em></td>
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<td>128</td>
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<td>8.0</td>
<td>4.0</td>
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<td>[256]</td>
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<td>[16]</td>
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<tr>
<td><em>Bacteroides thetaiotaomicron</em></td>
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<td>128</td>
<td>8.0</td>
<td>2.0</td>
<td>8.0</td>
<td>16</td>
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<tr>
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<td>128</td>
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<td><em>Prevotella melaninogenica</em></td>
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<td>128</td>
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<td>8.0</td>
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<tr>
<td><em>Prevotella disiens</em></td>
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<td>1.0</td>
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</tr>
<tr>
<td><em>Peptostreptococcus spp.</em></td>
<td>8.0</td>
<td>16</td>
<td>0.5</td>
<td>0.5</td>
<td>4.0</td>
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7.10. MEDICAL APPLICATIONS OF CERAGENINS

7.10.1. Contact lenses

Contact lenses are abiotic surfaces on which pathogens can form biofilms and proliferate, potentially leading to microbial keratitis, a serious condition that affects up to 25,000 Americans each year. AMPs are present in conjunctival fluid and provide the surface of the eye an innate immune function that controls bacterial growth. Efforts have been made to provide contact lenses with a comparable innate immune function by attaching AMPs to lenses, and this combination reduced the ability of bacteria to colonize lenses [118]. However, the relatively high cost of AMPs and concerns over thermal stability (lenses are generally autoclaved in the final stages of production) complicate the use of AMPs in contact lenses. The relatively low cost of ceragenins and the thermal stability of the ether-based compounds make them attractive alternatives to AMPs in providing an innate immune defense to contact lenses. In addition, ceragenins contain no chromophores (colorless), they are soluble in the polymers used to make lenses and they do not interfere with the polymerization processes involved in lens formation [119].

Two approaches were used in combining ceragenins and contact lenses. The first required appending a ceragenin with an acrylamide group, generating CSA-120 and allowing the ceragenin to participate in the radical polymerization steps that form lenses. The second involved formation of lenses in the presence of ceragenins, and in this approach ceragenins were able to elute from lenses.

Photo-initiated polymerization of acrylate groups is used to form hydrogel contact lenses. Acrylamide groups can participate in the polymerization process, and addition of CSA-120 to the reaction allowed covalent attachment of the ceragenin to the lens hydrogel. Up to 1.25 % CSA-120, relative to the dry mass of a lens, could be incorporated without causing phase separation in the hydrogel. When incorporated at this concentration in lenses, biofilm formation (S. aureus) was reduced by 3 logs after 24 h when tested in a 10 % bacterial growth medium. When 100 % medium was used, however, this inhibitory effect was lost, likely because the permanently bound ceragenin was covered by bacteria and bacterial debris.

To improve the duration of activity of ceragenins in contact lenses, a series of ceragenins were prepared in which the lengths of the lipid chain extending from bile acid were incrementally varied. The intent of these experiments was to match the hydrophobicity of the ceragenin to the hydrophobic domains in the lens hydrogel to limit elution of the ceragenin from the lens. With a shorter lipid chain, the ceragenin eluted too rapidly; however, with CSA-138, elution extended over weeks and provided antibacterial activity. At 1 % of the weight of dry lenses, CSA-138 inhibited the colonization of lenses by S. aureus for up to 30 days and by P. aeruginosa for up to 15 days (Figure 6) with daily inoculation.
with bacteria in fresh media. These results demonstrate that by tuning the properties of ceragenins to a hydrogel, the elution profile of the ceragenin can be controlled to provide long-lasting antimicrobial activity [119].

Figure 6. Bacterial population in nutrient media after 24 h incubation. Inoculated with *P. aeruginosa* ATCC 27853 (A), inoculated with *S. aureus* IBG 031 (B). Control lenses do not include any ceragenins. Lenses contained 1 % of selected ceragenins, relative to the dry weight of the lens. Every 24 h lenses were placed in fresh media and reinoculated.

7.10.2. Implanted medical device coatings

Implant-related infections affect thousands of patients each year [120], and active-release antimicrobial coatings have been designed in order to combat this issue. These coatings release antimicrobial agents into the surrounding
tissue and fluids with the intent of preventing biofilm formation on the device and infection at the implant site \[121,122\]. However, due to the presence of sessile organisms, if a biofilm forms on a device or the device is implanted in the presence of an established biofilm, most conventional antibiotics cannot fully protect implanted devices. An active-release coating containing ceragenins is an attractive option because ceragenins exhibit antibacterial activity against established biofilms, they do not lose effect in the presence of proteases and other enzymes, and they are easily incorporated into stable polymer coatings.

An active-release coating containing CSA-13 was first developed and described by Williams et al. \[123\] on fracture fixation plates. Investigation of the polymerization of a silicone coating showed that the incorporation of CSA-13 in particulate form (18 % w/w) had no impact on the physical properties of the coating and SEM observation revealed that the CSA-13 was distributed evenly throughout the coating. Of critical importance, when placed in aqueous solution, CSA-13 eluted from the coating over a span of 30 days. Furthermore, thermal stability testing of the coating demonstrated that coated fixation plates retained stability at elevated temperatures, providing further evidence that the CSA-13-silicone combination would be a viable option for the coating of implanted devices \[121,124\]. In order to evaluate this coating \textit{in vivo}, biofilms of methicillin-resistant \textit{S. aureus} (MRSA) were grown on a polymer mesh. After being allowed to reach population densities of over $10^9\,\text{CFU}\,\text{cm}^{-2}$, the mesh was implanted on the tibia of sheep and then immediately covered with a fracture fixation plate that was coated with CSA-13-silicone. Following inoculation, each sheep in the control group developed infection, while those that received fixation plates coated with CSA-13 were fully protected from developing infection over the course of the 12-week study. Investigation of the surrounding tissue showed that the coating was well tolerated, with no evidence of cytotoxicity or inhibition of wound healing. Remarkably, it was noted that there was an increase in bone healing in the presence of ceragenin.

The ability of this coating to prevent infection caused by planktonic bacteria was also evaluated \textit{in vitro} and \textit{in vivo} \[125,126\]. The coating was applied to porous titanium plug implants and placed in mouse models infected with MRSA. While control mice had to be euthanized per protocol shortly after the start of the experiment due to effects of infection, mice with CSA-13 coated implants were protected from infection for the duration of the 12-week study. Furthermore, CSA-13 released from implants did not damage skeletal attachment sites of the titanium plug implant compared to controls. Taken together, these \textit{in vivo} studies provide evidence that ceragenin-containing coatings can provide an effective antimicrobial function to implanted devices, allowing for the eradication of both planktonic and biofilm-associated bacteria.
7.10.3. Coated endotracheal tubes

Endotracheal tubes (ETTs) are another medical device that offer pathogenic bacteria and fungi an abiotic surface on which to proliferate, and microbial colonization of ETTs is associated with ventilator associated pneumonia (VAP) and prolonged stays in intensive care [127]. Use of ETTs with an innate immune-like function is expected to reduce VAP and healthcare costs substantially. To provide such a function to ETTs, CSA-131 was incorporated into a medical-grade hydrogel and evaluated for prevention of microbial colonization of ETT surfaces [128]. A key aspect in the design of the coating was to develop means of controlling elution of CSA-131 and to allow ethylene oxide sterilization of the coating without reaction with the ceragenin. Hydrochloride salts of ceragenins are thermally stable, allowing sterilization via autoclaving necessary for contact lenses; however, the hydrochloride salts allow some degree of reaction with ethylene oxide. In contrast, naphthylene disulfonate salts of CSA-131 (CSA-131 NDSA) were shown to be inert to ethylene oxide sterilization. Furthermore, the limited solubility of CSA-131NDSA provided a mechanism for controlled release from a hydrogel coating. Coatings were prepared with a milled form of CSA-131NDSA (sub-micron particle size) with coating thickness of 10 microns.

Coated ETTs were tested with daily inoculations of MRSA, *P. aeruginosa*, *K. pneumoniae*, *C. albican*, and *C. auris*, and coated tube segments remained uncolonized for up to 16 days (Figure 7). Coated ETTs were also effective against mixed-species inoculations, preventing biofilm formation for up to 4 days when inoculated with combinations of MRSA and *P. aeruginosa* or *P. aeruginosa* and *C. auris*. The surfaces of coated and uncoated ETT segments were visualized via SEM and showed no biofilm of mixed species of MRSA and PA01 or *C. auris* and PA01 after 48 h, whereas uncoated ETT segments were heavily colonized (Figure 8). To demonstrate the safety of coated ETTs, a pig intubation model was utilized. Pigs treated with coated ETTs showed no significant difference in tracheal or laryngeal inflammation and cilia loss was not significantly different between tests and controls. The coated ETTs also resulted in no systemic exposure of pigs to ceragenins as no CSA-131 was found in the blood with a detection limit of 5 µg mL⁻¹. The broad-spectrum activity of CSA-131 mimics that of AMPs and the hydrogel in which CSA-131NDSA is imbedded on coated ETTs provides a sustained release of the ceragenin. This system appears well suited for clinical use in preventing VAP in intubated patients [128].
Figure 7. Biofilms formed on uncoated (black bar) and coated (gray bar) tubes after the indicated number of incubation days. MRSA BAA-41 (A), K. pneumoniae ATCC 13883 (B), P. aeruginosa ATCC 47085 (C), C. albicans ATCC 90028 (D), C. auris CDC 0383 (E).

Figure 8. SEM images of biofilm of mixed species of MRSA and PA01 on an uncoated (A) and coated tube (B), biofilm of mixed species of PA01 and C. auris on an uncoated (C) and coated (D) tube after 48 h.
7.10.4. Bone fractures

Aside from their antimicrobial properties, AMPs have been shown to influence bone healing [129]. Considering this observation, the abilities of ceragenins to impact bone growth were determined. Selected ceragenins were tested in a rat femur-based model of bone regrowth to determine whether they would accelerate bone regrowth when used alone or in combination with bone morphogenic protein-2 (BMP-2), which is used clinically to accelerate bone regrowth. Of the ceragenins that were tested, CSA-90 displayed the most potent activity [130]. To determine if CSA-90 could positively impact bone regrowth while providing antibacterial protection of bone injury, a model of bone regrowth, complicated with infection, was used in a similar rat femur-based study. As anticipated, CSA-90 functioned to both prevent infection and potentiate the activity of BMP-2 in repairing and regrowing bone. In contrast, untreated rats had to be sacrificed per protocol within two weeks of fracture due to declining health. Because open fractures are very often complicated by infection, most often due to pathogens that dwell on the skin including S. aureus, the ability of ceragenins to both aid in bone regrowth while also serving to prevent infection at the fracture site could provide profound benefit to patients.

7.10.5. Imaging infections

AMPs and ceragenins display affinity toward bacterial membranes, and efforts have been made to leverage this property into selective association of AMPs or ceragenins, labeled with imaging agents, for use in imaging bacterial infections [131,132]. As further evidence of the affinity of ceragenins for bacterial membranes, CSA-124 was covalently attached to nanoparticles consisting of a silver shell with a maghemite core [114]. Imaging of the nanoparticles confirmed selective association with intact bacteria, with particular affinity for S. aureus. In vitro MRI studies further verified that these nanoparticles were able to adhere to S. aureus, indicating their potential to be used as contrast agents for use in imaging deep tissue infections [133].

Technicium (99mTc)-labeling of CSA-13 was also investigated as a means of imaging infections in mice. CSA-13 was chosen specifically because its multiple amine groups allow it to form stable complexes with the metal ion. Following direct infection of the thigh muscle in mice with S. aureus, the CSA-13-99mTc complex was administered. Imaging showed that the complex accumulated at the infection site and also in the kidneys [134]. Further efforts were made to alter the binding between the ceragenin and the technicium in order to allow the amine groups to remain free and available to form ionic interactions with bacterial membranes [135]. This modified complex has been shown to associate with S. aureus in in vitro studies.
7.10.6. Gastrointestinal diseases

*Helicobacter pylori* is carried by about half of the world’s adult population. While carriers are often asymptomatic, infection can lead to severe ulceration and gastritis, and is associated with gastric adenocarcinoma. The emerging threat of antibiotic-resistant strains led Leszczynska *et al.* [136] to test whether ceragenins could be used as a viable treatment option. They found that CSA-13 displayed significantly lower minimum bactericidal concentrations (MBC) against a variety of *H. pylori* clinical isolates as compared to LL-37. Furthermore, under simulated gastric conditions in the presence of low pH and pepsin, CSA-13 retained its bactericidal activity while the activity of LL-37 was lost. Interestingly, *H. pylori*, with its unique defense mechanisms of incorporating host cholesterol into its membrane and activity through its HefC efflux pump, showed some resistance to ceragenins, which was based on the presence of cholesterol in the bacterial membrane [137].

The impact of CSA-13 in gastrointestinal infections caused by *Clostridium difficile* has been studied in a mouse model [138]. Oral administration of an active formulation of CSA-13 containing Eudragit and methylcellulose resulted in CSA-13 release in the terminal ileum and colon where the environmental pH is alkaline. The inhibition of *C. difficile* spore germination and cell viability by CSA-13 was measured at 4 µg mL\(^{-1}\). Both subcutaneous and oral administration of CSA-13 decreased the concentration of *C. difficile* in fecal samples of mice, however *Peptostreptococcaceae* bacteria abundance increased suggesting that CSA-13 suppresses *C. difficile* via modulation of intestinal microbiota. Therefore, other microbial species and the balance of the gut microbiota should be considered with regard to further therapeutic application of CSA-13 in gastrointestinal diseases. According to the observations from this study, the protective action of CSA-13 could be generated through the direct suppression of *C. difficile*, by lowering the levels of pro-inflammatory metabolites (endocannabinoids), or by increasing the levels of protective metabolites (citrulline, 3 aminoisobutyric acid, retinol, and ursodeoxycholic acid) in the intestine [138].

7.11. CONCLUSIONS

The central and critical roles that AMPs play in innate immunity have been well documented and include broad-spectrum antimicrobial activities, the ability to sequester microbial endotoxins and thereby inhibit inflammatory processes and additional activities that accelerate wound healing. Recognition of these roles has led to intense research of AMPs; for example, thousands of papers have been published describing activities of the human cathelicidin LL-37. The potential for therapeutic use of AMPs has also been well
recognized; however, issues related to clinical use have also emerged: “AMPs possess some limitations that hamper their clinical and commercial development such as high production costs, potential toxicity, susceptibility to proteases (also in the wound fluid), and unknown pharmacokinetics” [139]. By abandoning peptide structure, while retaining overall morphology, ceragenins provide a means of reproducing the beneficial properties of AMPs without incurring many of the issues hampering clinical use of AMPs.

Current studies with ceragenins have focused on applications in tissues that express relatively high amounts of AMPs, and ceragenins have proven to be well tolerated in these tissues. However, less work has been done with potential systemic exposure to ceragenins. Use of ceragenins in coatings of medical devices and at bone fractures could lead to wider exposure, but no adverse events have been observed. Intraperitoneal injection of CSA-13 into mice in an infection model showed efficacy without measured toxicity [140]. The possibility of systemic use of ceragenins exists, but the membrane-targeting activity of ceragenins argues that their most attractive uses will be in localized applications. With successful demonstration of efficacy in vitro in multiple animal models, it is anticipated that ceragenins will continue development toward clinical use and eventual applications in replacing or augmenting the activities of endogenous AMPs.
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Ceragenins as non-peptide mimics of endogenous antimicrobial peptides


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SMALL MOLECULES TARGETING AT THE BACTERIAL CELL DIVISION PROTEIN FtsZ AS POTENTIAL ANTIMICROBIAL AGENTS

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Chapter 8

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8.1. INTRODUCTION

8.1.1. Antibiotics and antimicrobial resistance

Since the discovery of the first antibiotic, penicillin, by Alexander Fleming in the 1930s [1], the following 40 years was the “golden era” of antibiotic research and most of the antibiotics currently in use were discovered and developed in that period [2]. The discovery of antibiotics was once regarded as the ultimate victory of the battle against bacterial infections but unfortunately the development of antimicrobial resistance to common antibiotics implies that this battle is endless. Nowadays, the treatment of bacterial infections has become more difficult because bacteria can develop resistance to antibiotics at an alarming speed [1-4]. Vancomycin-resistant Enterococcus faecium (VREF) and methicillin-resistant Staphylococcus aureus (MRSA) are two typical representatives of gram-positive bacteria that are resistant to conventional antibiotics including vancomycin and methicillin [5,6]. This situation is even more alarming in gram-negative bacteria. Bacteria express New Delhi metallo-beta-lactamase-1 (NDM-1) are known to be highly resistant to most antibiotics. So far, only colistin and tigecycline are still effective against these superbugs [7,8]. Therefore, discovery of the next-generation antimicrobials with novel mechanisms of action is urgently needed [4,9].

8.1.2. Bacterial cell division proteins as potential new drug targets

Cell division is an essential process for bacterial survival. During bacterial cell division, over 30 proteins assemble orderly to form the ‘divisome’ complex in such a way that the division process is controlled in a systematic manner. Many proteins involved in bacterial cell division are potential drug targets in antibiotic development. Among the bacterial cell division proteins, the filamenting temperature-sensitive mutant Z (FtsZ) is the first protein that assembles and initiates the cell division process. FtsZ is highly conserved in a wide range of bacteria [10-13]. During bacterial cell division, FtsZ assembles into a highly dynamic cytoskeleton scaffold called the Z-ring by undergoing guanosine-5’-triphosphate (GTP)-dependent polymerization, forming head-to-tail protofilaments and assembling into bundles at the site of septum formation [14-16]. Subsequently, downstream proteins that are responsible for septum formation and invagination of cell membrane are recruited by FtsZ to complete the bacterial cell division [17,18]. The high conservation and functional importance of FtsZ in bacterial cell division render it as an attractive target for the development of novel antibacterial agents.
8.2. STRUCTURE OF FtsZ AND ITS FUNCTIONS

8.2.1. The structure

In FtsZ protein (Figure 1), there are two domains (the C-terminal and N-terminal domains) [19,20] separated by a central core helix (H7 helix) and a synergy loop (T7 loop). The N-terminal domain contains a nucleotide-binding pocket, while the C-terminal domain and T7 loop contain residues essential for the GTPase activity of the protein. During polymerization of FtsZ protein, an active site for GTP hydrolysis is formed by the nucleotide-binding site of one FtsZ monomer, and the T7 loop of another FtsZ monomer. In addition, at the tail of the long C-terminal extension, there are some functional sites which enable the FtsZ protein to distinguish other accessory proteins during cell division [21-24].

Figure 1. The structure of FtsZ monomer: the N-terminal domain (blue-colored region), the C-terminal domain (red-colored region), and GDP in the nucleotide binding site (green-colored molecule). The yellow-colored molecule is PC190723, which is located at the interdomain cleft between H7 helix and C-terminal domain (PDB entry 4DXD) [25].
FtsZ is a structural homolog of the eukaryotic cytoskeleton protein tubulin. This is probably due to their similar structures and functions. To compare their structure, FtsZ and tubulin have a common fold and consisting of N-terminal and C-terminal domains linked by an $\alpha$-helix (H7) [19,26]. The conserved FtsZ residues and tubulin are both found in the nucleotide-binding region and this region is correlated to the formation of protofilament [19,27]. Tubulin is able to polymerize into microtubules and its function is to migrate the genetic materials to the poles of a dividing cell. The function of FtsZ is to polymerize to form Z-ring for controlling the division of a bacterial mother cell into daughter cells [26,28,29]. Furthermore, just as the microtubule assembly is influenced by microtubule associated proteins, the stability of FtsZ polymerization is controlled in vivo by bacterial cell division proteins such as FtsA, ZipA, and ZapA [28,30-34].

Although certain common features are found in FtsZ and tubulin, there are only 7% sequence identity at the protein level of the two proteins [35]. In fact, substantial differences can be found in the binding modes of their nucleotides [36,37]. Moreover, the amino acid sequence of C-terminus and the $\alpha$-helices contents are not the same in the two proteins [38]. In addition, tubulin forms $\alpha$ and $\beta$ heterodimers of different polarity, whereas the FtsZ polymer only contains FtsZ monomers in one form [39]. Furthermore, FtsZ protofilaments are different from the longitudinal associated tubulin subunits in that they associate with each other laterally to form an arc-shaped structure [40]. The differences in the structures of FtsZ and tubulin allow the development of new antibacterial agents with low eukaryotic cell cytotoxicity by targeting at the FtsZ protein.

### 8.2.2. Function and dynamics

Bacterial cell division starts with FtsZ polymerization with GTP in the middle of the cell. The polymers formed at the center of the cell then further develop bi-directionally and assemble into a helical structure known as Z-ring, which is highly dynamic [40-43]. Subsequently, various downstream proteins are recruited to the Z-ring structure for divisome complex formation. The Z-ring contracts to contribute to the septum formation in the final process of cell division (Figure 2) [44,45].

Both monomeric and oligomeric forms of FtsZ can be found in the cytoplasm of bacteria. For a single *E. coli* cell, there are nearly 15,000 copies of FtsZ and in the entire cell cycle the FtsZ concentration almost remains constant [30,46]. Interestingly, at a given time, there are only about 30% of FtsZ taking part in the Z-ring formation. These participated FtsZ molecules undergo rapid exchange with other FtsZ molecules in the cytoplasm at a half-life of approximately eight seconds [47,48]. Therefore, the resulting Z-ring is a dynamic structure balanced between assembly and disassembly [49].
Figure 2. (A) Formation of protofilaments from FtsZ monomers polymerization in the presence of GTP; (B) Lateral interactions between protofilaments to form the Z-ring; the Z-ring is attached to the cell membrane via interaction with FtsA and ZipA; dynamic exchange of FtsZ monomers between the Z-ring and cytoplasm; (C) Invagination of cell membranes resulting from the contraction of the Z-ring; (D) Z-ring dissipates and the cell division is completed.

The FtsZ assembly process is started under the conditions that separation of nucleoids and replication of DNA are completed [30], and the required GTP concentration is above a critical concentration of 0.5–1 μM [50-52]. When these external conditions are realized, FtsZ monomers bind with GTP and undergo cooperatively assembly in a head-to-tail manner to form protofilaments that are straight single-stranded protein filaments (4–5 nm in width) [53]. After the assembly, GTP is sandwiched in between the two adjacent FtsZ monomers. At the same time, the activity of GTPase is also triggered to hydrolyze GTP [54-56]. Then the protofilaments merge through the lateral interactions into condensates such as pairs, bundles, and sheets. As a result, the highly dynamic Z-ring structure is formed [36,50,57,58]. During polymerization, the T7 loop of FtsZ is pushed into the nucleotide-binding region of another FtsZ monomer. The catalytic residues of the T7 loop are positioned near the phosphate group of GTP to promote the hydrolysis reaction [59]. The protofilaments change their conformation from straight to curved when GTP is hydrolyzed to guanosine diphosphate (GDP) [60]. The hydrolysis of GTP provides the required energy for cell division. The conformation change of FtsZ protofilaments is also believed to transfer the mechanical force to the cell membrane for its invagination before cell division completion [60,61]. In addition, the process of GTP hydrolysis is associated to the de-polymerization of FtsZ [49]. In recent years, it has been reported that in some FtsZ-mutant cells, even when there is no detectable GTPase activity, the mutant cells can still show cell division ability and take the physiological role of division. For this reason, the hydrolysis reaction of GTP is not considered as
Small molecules targeting at the bacterial cell division protein FtsZ ...

the only energy source for the construction of the septum but is regarded as a promotive element in the symmetric invagination of cell division [62,63].

The FtsZ subunits assembly can be observed at the mid-cell in a temporally regulated stage-specific manner. MinCDE system and Noc (B. subtilis)/Sima (E. coli) are the regulatory factors to ensure the location of FtsZ assembly between the segregated chromosomes in the middle region of the cell but not at the cell poles [18,64]. It is believed that protofilaments annealing leads to the lateral interactions for most protofilaments [64]. It is also suggested that the lateral interactions are probably caused by the electrostatic forces of ions from the protofilaments [58]. Currently, the mechanism of protofilament assembly remains to be in dispute and needs to be further investigated [65].

For Z-ring formation, the FtsZ-interacting proteins play a very vital role in the process. In particular, FtsA and ZipA that bind to the C-terminal domain are the most essential proteins for proper cytokinesis [31,32]. FtsA is conserved in most bacteria and is able to displace ZipA. It is therefore regarded as a more important factor than ZipA for Z-ring integrity [31,32]. The formation of Z-ring is also influenced by some stabilizing proteins including SepF and ZapA, and destabilizing proteins like SulA and MinCD [42], as well as other factors such as glutamate [66], calcium [67], pH value, and the ionic strength [68].

8.3. FtsZ INHIBITORS

With the rising of drug-resistant bacteria, the development of new antibacterial agents with novel mechanism of action is highly desirable. FtsZ is a well-studied protein and is a potential new drug target. Several characteristics render FtsZ a potential target for the development of new antibacterial agents: (i) it plays an essential role in bacterial cell division [45]; (ii) the structure and function of FtsZ are conserved across bacterial species [17,20]; (iii) it is absent in eukaryotes [69,70]; (iv) its structural and biological properties are well studied. So far, there are no drugs on the market targeting at FtsZ, but many researchers have made great efforts in studying FtsZ targeting compounds and their studies revealed that FtsZ inhibitors can lead to bacterial cell death via inhibition of cell division [71-74]. Most of these inhibitors will be discussed in the following sections.

8.3.1. Natural products and their derivatives

8.3.1.1 Berberine and its derivatives

Berberine (1 in Figure 3) is a common natural alkaloid from Berberis. In 2008, Dasgupta and co-workers reported that berberine interacted with E. coli FtsZ by inhibiting the GTPase activity (IC$_{50}$ 16 μM) and assembly (IC$_{50}$ 10 μM) of
FtsZ in a dose-dependent manner. Molecular modeling study suggested that the binding site of berberine overlapped with the GTP binding pocket in \textit{E. coli} FtsZ [75].

Using a method of \textit{in silico} structure-based design, Wong and co-workers synthesized a number of 9-phenoxyalkyl berberine derivatives, which were suggested to bind to the C-terminal interdomain cleft of \textit{S. aureus} FtsZ. These derivatives inhibit the growth of MRSA and VRE with minimum inhibitory concentrations (MICs) of 2–8 $\mu$g mL$^{-1}$ and 4–16 $\mu$g mL$^{-1}$, respectively. These compounds also exhibit moderate antimicrobial activity against the gram-negative strains such as \textit{E. coli} with MIC of 32–128 $\mu$g mL$^{-1}$. Among these derivatives, compound 2 (Figure 3) possessed the most potent antibacterial activity and inhibits the growth of MRSA and VRE with MICs of 2 and 4 $\mu$g mL$^{-1}$, respectively. In addition, it can inhibit FtsZ polymerization and GTPase (IC$_{50}$ 38 $\mu$g mL$^{-1}$) in a dose-dependent manner. The results of transmission electron microscopy (TEM) images revealed that the compound reduced the size and thickness of FtsZ polymers significantly [76].

\subsection*{8.3.1.2 Cinnamaldehyde and its derivatives}

Cinnamaldehyde (3 in Figure 3) is a natural aromatic compound derived from the stem bark of \textit{Cinnamomum cassia}. It inhibits \textit{E. coli} FtsZ GTPase activity and polymerization with IC$_{50}$ values of 5.81 $\mu$M and 6.86 $\mu$M, respectively. Molecular modeling and STD-NMR spectroscopy showed that cinnamaldehyde binds to FtsZ around the T7 loop in the C-terminal region [77].

Ma and co-workers synthesized serials of cinnamaldehyde derivatives and tested their antibacterial activity against Gram-positive and -negative bacteria. Some of them exhibited cell division inhibitory effect on \textit{S. aureus} ATCC25923 in the concentration range of 0.25–4 $\mu$g mL$^{-1}$. Compounds containing a 2-methylbenzimidazolyl substitution at 1-position and 2,4-dichlorophenyl at the 3-position exhibited the best activity (Compound 4). The results of biological assays revealed that the compounds can inhibit polymerization and GTPase activity of \textit{S. aureus} FtsZ in a dose-dependent manner [78].

\subsection*{8.3.1.3 Chrysophaeintins}

Chrysophaeintins A-H are natural products isolated from the marine chrysophyte alga \textit{Chrysophaeumtaylori}. Bewley and co-workers found that chrysophaeintins are effective against several Gram-positive bacterial strains. Among them, chrysophaeintin A (5 in Figure 3) showed the best antibacterial activity against many drug-resistant bacteria. For examples, the MICs of this compound against MRSA and VRE are 1.5 $\mu$g mL$^{-1}$ and 2.9 $\mu$g mL$^{-1}$, respectively. In addition, chrysophaeintin A was found to inhibit the GTPase activity of \textit{E. coli}
FtsZ with \(IC_{50}\) of 6.7 \(\mu\text{g mL}^{-1}\) and block FtsZ polymerization. STD-NMR experiment and competition assay indicated that chrysophaentin A interacted with FtsZ at the GTP binding site [79]. As a continued study, Bewley and co-workers synthesized hemi-chrysophaentin and reported that the reaction mechanism is similar to that of chrysophaentin A. In the GTPase assay, chrysophaentin fragment (Compound 6) exerted inhibition effect on the GTPase activity of \(S.\ aureus\) FtsZ with \(IC_{50}\) of 38 ± 9 \(\mu\text{M}\) and \(E.\ coli\) FtsZ of 37 ± 7 \(\mu\text{M}\). The result of fluorescence anisotropy test indicated a competitive binding of 6 with GTP in the nucleotide binding region [80].

### 8.3.1.4 Curcumin

Curcumin (7 in Figure 3) is a dietary polyphenolic compound extracted from the rhizomes of \textit{Curcuma longa}. In Indian cooking, curcumin is commonly used as a spice and a coloring agent and is known to have antibacterial activity against a broad range of bacterial pathogens. Rai and co-workers reported that curcumin decreased light scattering intensity of FtsZ protofilaments in a dose-dependent manner, indicating its inhibitory effect on the FtsZ polymerization. The results of GTPase assay indicated that the interaction of curcumin with FtsZ enhanced the GTPase activity but destabilized the FtsZ polymers [81]. Using computational docking, Roy and co-workers identified the possible binding sites of curcumin in \(E.\ coli\) FtsZ and \(B.\ subtilis\) FtsZ. Curcumin was predicted to interact with several amino acid residues in the GTP binding pocket [82].

### 8.3.1.5 Viriditoxin

Viriditoxin (8 in Figure 3) originates from \textit{Aspergillus viridinutans}. It inhibits GTPase activity of \(E.\ coli\) FtsZ with \(IC_{50}\) of 7.0 \(\mu\text{g mL}^{-1}\) and FtsZ polymerization with an \(IC_{50}\) value of 8.2 \(\mu\text{g mL}^{-1}\). Morphological study revealed that viriditoxin can cause the elongation of \(B.\ subtilis\) cells. Moreover, viriditoxin displays strong antibacterial effect on a number of drug-resistant Gram-positive pathogens, like various strains of \(S.\ aureus\) (MIC 4–8 \(\mu\text{g mL}^{-1}\), \(E.\ faecalis\) and \(E.\ faecium\) (MIC 2–16 \(\mu\text{g mL}^{-1}\)). The induction of FtsZ expression in bacterial cells could increase the MIC value, suggesting that viriditoxin targets with FtsZ in these bacterial strains [83].

### 8.3.1.6 Coumarins

Coumarins are phytochemicals consisting of a benzopyrone core. Coumarin (9), scopoletin (10), and daphnetin (11) show moderate inhibitory effects on the polymerization and GTPase activity of FtsZ (Figure 1). Scopoletin inhibits
FtsZ polymerization with an IC$_{50}$ of 41 μM and daphnetin with an IC$_{50}$ of 73 μM. The GTPase activity was inhibited by scopoletin and daphnetin with IC$_{50}$ values of 23 μM and 57 μM, respectively. Molecular modeling studies of FtsZ and coumarins suggested that coumarins may bind to the T7 loop of FtsZ [84]. Furthermore, Doble and co-workers suggested that coumarins exhibit antibacterial activity against *M. tuberculosis* H37Rv through inhibiting polymerization and GTPase activity of FtsZ [84].

### 8.3.1.7 Plumbagin

Plumbagin (12 in Figure 3) is a naphthoquinone derivative from the root of *Plumbago zeylanica* and is known to possess antibacterial activities against a number of bacteria. Panda and co-workers reported that plumbagin can disturb Z-ring formation in *B. subtilis* 168 cells and cause cell elongation without an apparent effect on nucleoid segregation. Their results indicate that the FtsZ assembly process may be inhibited by plumbagin. Biochemical studies showed that plumbagin can effectively inhibit the GTPase activity of FtsZ. For instance, 24 μM plumbagin is able to inhibit 58% of the GTPase activity. Moreover, plumbagin inhibits the FtsZ polymerization of *B. subtilis* in a dose-dependent manner. Thus, 2, 5, and 10 μM of plumbagin can reduce FtsZ polymerization by 26%, 33%, and 45%, respectively. Computational analysis and mutagenesis assay suggested that the binding site of plumbagin may locate near the C terminal of *B. subtilis* FtsZ and residues D199 and V307 may be involved in the interaction between plumbagin and FtsZ [85].

### 8.3.1.8 Phenylpropanoids

Phenylpropanoids are the secondary metabolites produced by plants against pathogens. These compounds possess moderate antimicrobial activities [86]. Eight phenylpropanoids were tested against *E. coli* FtsZ and were found to disturb GTPase activity of FtsZ in vitro. For example, 100 μM chlorogenic and ferulic acids inhibited the GTPase activity by 46 and 34%, respectively. Caffeic and p-coumaric acid reduce the GTPase activity by 23 and 29%, respectively. Light scattering assays revealed that these compounds inhibit FtsZ polymerization in a dose-dependent manner. Chlorogenic acid, which is most active among the phenylpropanoids, has an IC$_{50}$ of 69.55 ± 3.6 μM. Caffeic acid, 2,4,5-trimethoxycinnamic acid, p-coumaric acid, and cinnamic acid have IC$_{50}$s of 105.96 ± 6.3 μM, 148.59 ± 4.3 μM, 189.53 ± 3.7 μM, and 238.91 ± 7.1 μM, respectively. 3,4-Dimethoxycinnamic acid, 2,4,5-trimethoxycinnamic acid, and eugenol were the least active compounds with IC$_{50}$ > 250 μM. Furthermore, morphological studies showed that these compounds can inhibit the bacterial cell division and induce cell elongation of *B. subtilis* 168 cells [87].
8.3.1.9 Dichamanetin and its derivative

Dichamanetin (13 in Figure 3) and its derivative, 2’’-hydroxy-5’’-benzylisouvarinol-B (14 in Figure 3), are polyphenolic compounds extracted from *Uvariachamae* and *Xylopia Africana*, respectively [88]. These natural products display antibacterial activity against many bacteria. Particularly, they possess potent antibacterial activity against *S. aureus* with MIC values of 0.8 μg mL⁻¹ and 1.16 μg mL⁻¹, respectively [88,89]. As a continuation of mechanistic study, Urgaonkar and co-workers evaluated the effects of dichamanetin on the GTPase activity of *E. coli* FtsZ and reported the inhibition IC₅₀ values are 12.5 μM and 8.3 μM, respectively [89].
Figure 3. The structures of FtsZ inhibitors from natural source
8.3.2. Synthetic small organic molecules

8.3.2.1 Benzamides

3-Methoxybenzamide (3-MBA) (Figure 4) was reported a FtsZ inhibitor leading to cell elongation in *B. subtilis* [90]. In view of the advantages of 3-MBA such as low molecular mass and good ligand efficiency, Czaplewski and co-workers established a medicinal chemistry program to search for various 3-MBA derivatives [91]. They performed structure–activity relationship (SAR) study with 3-MBA based on more than 500 3-MBA congeners and eventually identified PC190723 (16) which possesses much better potency than the other 3-MBA derivatives. PC190723 exhibits effective antibacterial activity against *B. subtilis* and various strains of *S. aureus*, such as MRSA and multidrug resistant *S. aureus* (MDRSA). The MIC values were found to be as low as 1 μg mL\(^{-1}\). Moreover, PC190723 inhibits the GTPase activity of *S. aureus* FtsZ in a dose-dependent manner with an IC\(_{50}\) value of 55 μg mL\(^{-1}\). In the cell morphology study, rod-shaped *B. subtilis* or spherical *S. aureus* treated with PC190723 exhibited cell elongation or enlargement. In addition, PC190723 was found to disturb Z-ring formation in the *B. subtilis* cells. Docking study showed that PC190723 binds to the interdomain cleft between C-terminal and H7 helix, which is consistent with the result of a mutation study [91]. Andreu and co-workers found that PC190723 inhibited GTPase activity of *B. subtilis* FtsZ through inducing FtsZ polymers into straight bundles and ribbons. The result suggested that PC190723 inhibit FtsZ via the stabilization of the FtsZ polymerization [92]. Lumb and co-workers also conducted some structural and biochemical analyses of *S. aureus* FtsZ with PC190723. The study pointed out that PC190723 shifted the structural equilibrium of FtsZ to a conformation with high-affinity [93]. From the co-crystal structure of PC190723 interacted with *S. aureus* FtsZ, the C-terminal domain was found to move in such a way that stabilization of FtsZ protofilaments was better than that of FtsZ monomers [93]. Therefore, PC190723 represents a new type of inhibitor targeting at FtsZ with high efficiency and possibility for further structural optimization.

Stokes et al. developed some PC190723 analogs (Figure 4), which bear a phenyloxazole group to replace the thiazolopyridine group and a substituted methylene group connecting to the oxazole and benzamide moieties. The (4,5-disubstituted oxazolyl)-CH(R)-O-benzamides are able to enhance the antibacterial activity significantly against wild type *S. aureus* (Compound 17 in Figure 4, MIC is 0.03 μg mL\(^{-1}\)). The C5 position of the oxazole moiety can be modified with alkyl, aryl or halogen substituents to achieve higher potency. In this series of compounds, 5-bromo- and 5-chlorooxazolyl analogs exhibit high activity against a mutant strain of *S. aureus*, G196A [94,95]. To increase the pharmacokinetic parameters of the compounds, polar groups such as alcohol, amine, carboxylic acid and heterocycles were incorporated to the pseudo-benzylic position (−CH(R)−O− moiety). These polar groups increase the solubility and the metabolic stability of the compounds. However, when a gem-
dimethyl group was introduced at the pseudo-benzylic position, it results in loss of activity, which suggests that chirality is a crucial factor in antibacterial activity. The enantiomers of compound 18 in Figure 4 (having \(-\text{CH(CH}_2\text{OH})\text{-O}\) group) after isolated with chiral chromatography were tested for antibacterial activity. Surprisingly, (R)-(\(+\))-enantiomer shows 128 times more effective than the (S)-(\(-\))-enantiomer. In order to further increase the solubility of compound 18, a prodrug 19 was synthesized. The solubility of 19 was 2-fold higher than its parent compound [94,95]

To further increase the pharmacological properties of PC190723, Pilch and co-workers synthesized 1-methylpiperidine-4-carboxamide TXY541 (20, Figure 4), which is a prodrug of PC190723. The compound was effective against MRSA [96]. Its solubility in an aqueous acidic vehicle (10 mM citrate, pH 2.6) is about 143 times higher than that of PC190723. The compound also showed efficacy \textit{in vivo} via intravenous and oral administration using \textit{S. aureus} infectious mice model. TXA541 showed a bactericidal mode of action against MRSA with a MBC/MIC ratio of 2. TXA707 (21) was also developed by adding a trifluoromethyl group instead of the chloro group in TXY541 and TXA709 (22) to overcome the CYP-mediated dechlorination/oxygenation [97]. The addition of trifluoromethyl group was able to improve metabolic stability substantially. The MIC value of TXA707 was 1 \(\mu\)g\,\text{mL}^{-1} against MRSA and VRSA. The \(t_{1/2}\) of TXA707 following intravenous administration of TXA709 was 3.65 h, while the \(t_{1/2}\) of PC190723 following the intravenous administration of TXA541 was 0.56 h. TXA709 thus displayed better pharmacokinetic properties by keeping its strong antibacterial activity and its efficacy \textit{in vivo}. In a study of the mice model infected with MRSA ATCC 33591, a 2-log reduction in bacterial CFU can be found in the model treated with 120 or 160 mg\,kg\(^{-1}\) of TXA709 administered orally, while only about 0.5-log reduction was found when orally administered 200 mg\,kg\(^{-1}\) of PC190723 [25,97].
8.3.2.2 Arene-diol digallate derivatives

In a search for small molecular inhibitors that can compete with GTP in FtsZ protein, Ruiz-Avila et al. selected a series of small molecules from virtual screening hits, literature and in-house compounds by docking into the *B. subtilis* FtsZ GTP binding site. The hits obtained were further examined by *mant*-GTP anisotropy. The results showed that UCM05 (23 in Figure 5), UCM44 (24 in Figure 5), and UCM53 (25 in Figure 5) can inhibit bacterial growth with MIC values of 100 μM, 25 μM, and 13 μM respectively. The binding modes of these compounds suggest that the inhibitory effects were contributed from the phenolic groups and the naphthalene core that occupied the phosphate and nucleic base binding sites of GTP. Moreover, all the three compounds can effectively induce cell elongation and disturb Z-ring formation in *B. subtilis* cells [98]. Based on the structure of these compounds, a number of small molecule inhibitors were synthesized and their activities against bacteria and FtsZ protein were evaluated. The most potent compound 26 (Figure 5) displays a strong binding affinity with FtsZ ($K_d$ 0.5 μM) and antibacterial activity against MRSA (MIC value of 7 μM). Most of arene-diol digallates at
100 μM show little inhibition on tubulin polymerization, suggesting high selectivity to FtsZ [99].

8.3.2.3 Pyrimidine-quinuclidine derivatives and analogs

To identify hit compounds targeting at the GTP binding site of FtsZ, Wong and co-workers carried out a structure-based virtual screening of over 20,000 compounds, which contain natural products and their semisynthetic derivatives using the crystal structure of Methanococcus jannaschii FtsZ [100]. The ten top-ranked compounds from the screening were further tested for their GTPase inhibitory effect in vitro and antibacterial activity against pathogens. Among these compounds, compound 27 (Figure 5), which contains a pyrimidine-quinuclidine moiety as the core structure, exhibits moderate GTPase inhibitory effect against S. aureus FtsZ (IC₅₀ 317 μM) and antibacterial activity against S. aureus and E. coli with MIC values of 449 μM and 897 μM, respectively. The compound was selected as the hit compound for further in silico optimization. A homology modeling of S. aureus FtsZ was used for screening new pyrimidine-quinuclidine derivatives. The screening generated compound 28 which displays significantly activity improvement in the biological tests. The GTPase inhibitory effect (IC₅₀ 37.5 μM) and antibacterial activity against S. aureus (MIC 24.6 μM) and E. coli (MIC 49.6 μM) of 28 were more than 10 times higher compared to that of 27 [100]. In addition, these derivatives were reported to selectively inhibit FtsZ over tubulin and restore susceptibility of MRSA (ATCC BAA-41) to β-lactam antibiotics [101].

Employing pyrimidine-quinuclidine scaffold as a template, Wong and co-workers successfully synthesized 99 amine-linked 2,4,6-trisubstituted pyrimidine derivatives and investigated their biological activities. The results of antimicrobial susceptibility assay against S. aureus strains confirmed that these compounds possessed potent anti-staphylococcal activity (MIC ranged from 3–8 μg mL⁻¹). Biochemical studies of compound 14av_amine16 (29) by STD-NMR, light scattering and GTPase activity assay with S. aureus FtsZ revealed that it interacts with the FtsZ protein via inhibiting polymerization and GTPase activity of FtsZ. Furthermore, mislocalization of the Z-ring and filamentous cell phenotype of B. subtilis suggested that 29 is a FtsZ targeting compound. The compound also exhibits low frequency of resistance on S. aureus and low toxicity against Galleria mellonella larvae [102].
Small molecules targeting at the bacterial cell division protein FtsZ ...

8.3.2.4 Benzopyridines

LaVoie and co-workers studied the effects on the antibacterial activity of a number of berberine derivatives having an aryl substituent located at the 2- and 12-position [103]. The results suggested that addition of biphenyl substituent at either 2- or 12-position of berberine derivatives dramatically improves its antibacterial activity against S. aureus and E. faecalis. Compound 30 with a 2-biphenyl substituent (Figure 6) exhibited the best activity against MRSA (ATCC 33591) and vancomycin-resistant E. faecalis with MIC values of 0.5 μg mL$^{-1}$ and 2 μg mL$^{-1}$ respectively. Moreover, in the light scattering assay,
was found to significantly increase the light signal in compared with the control, indicating its stimulation of FtsZ polymerization [103].

It has been reported that the addition of a hydrophobic substituent to sanguinarine can enhance the antibacterial activity dramatically [103]. LaVoie and co-workers further simplified their common scaffolds to produce a new 3-phenylisoquinoline core. The derivatives based on 3-phenylisoquinoline and 3-phenylisoquinolinium core were thus synthesized for antibacterial activity evaluation [104]. Their results indicated that the quaternary isoquinolinium derivatives exhibited higher activity than the corresponding non-quaternary isoquinolines. In addition, the lipophilicity of the substituent at the 3'-position contributed to the antibacterial efficacy. Compound 31 (in Figure 6) exerted potent inhibition of MRSA and VRE with MIC values of 1 μg mL⁻¹ and 4 μg mL⁻¹ respectively. Moreover, the binding properties of these compounds to S. aureus FtsZ were monitored by intrinsic fluorescence spectroscopy. The dissociation constants were found to be in the range of 1 to 10 μM. In addition, this compound showed little effect on the mammalian tubulin and low cytotoxicity to mammalian cells [104].

Based on the above-mentioned study of quinoline derivatives, Sun et al. studied the antibacterial activity of N-methylbenzoindolo[3,2-b]-quinoline and N-methylbenzofuro[3,2-b]-quinoline derivatives, as well as a thiazole orange quinolin-1-ium iodide derivative[105,106]. The results revealed that these compounds possess potent antibacterial activity against bacterial strains including MRSA and VRE. The results of biological study suggested that these compounds disrupt the dynamic assembly of FtsZ protein and Z-ring formation through inhibiting GTPase activity of FtsZ [105,106].

8.3.2.5 Rhodanine derivatives

OBTA (3-{5-[4-oxo-2-thioxo-3-(3-trifluoromethylphenyl)-thiazolidin-5-ylidene-methyl]-furan-2-yl}-benzoic acid, 32 in Figure 6) was found to enhance FtsZ assembly in an in vitro screening of 81 compounds having 29 different structural scaffolds on FtsZ polymerization [107]. OBTA can reduce the GTPase activity of FtsZ and improve the polymerization of FtsZ. The binding affinity study revealed that OBTA interacted with FtsZ at a dissociation constant of 15 μM. Furthermore, OBTA induced bacterial cell elongation and disturbed Z-ring formation in bacteria. In addition, OBTA possesses a potent antibacterial activity, and shows little cytotoxic effects on mammalian cells [107]. The results indicate that OBTA may be a potential lead compound for further development of FtsZ inhibitors.

Panda and co-workers screened a library of 151 rhodanine derivatives in an attempt to discover new FtsZ inhibitors [108]. Among these derivatives, CCR-11 (33 in Figure 6) could exert inhibition on B. subtilis with an MIC of 3 μM and induce the elongation of B. subtilis cells. The results of biological assays showed that CCR-11 could inhibit the GTPase activity and polymerization of FtsZ. The
Small molecules targeting at the bacterial cell division protein FtsZ ...

influences on Z-ring nucleoids and membrane were further examined in elongated *B. subtilis*. The results showed that CCR-11 can perturb Z-ring formation effectively without any impairment of nucleoids and membrane. From the docking study, CCR-11 is predicted to bind to a cavity near the T7 loop. The binding energies estimated theoretically are comparable to the binding constant (1.5 μM) determined experimentally. With regard to the selectivity of this compound, the effects on mammalian cells showed that the cytotoxicity of CCR-11 is low [108].

8.3.2.6 Pyridopyrazine and pyrimidothiazine analogs

Since FtsZ shares some common features with eukaryotic tubulin, Reynolds and co-workers screened FtsZ-targeting compounds with similar structures to the anti-tubulin molecules in order to find anti-tuberculosis drugs [109]. Their study led to the discovery of 34 and 35 (Figure 6) which showed active effects on the polymerization of *M. tuberculosis* FtsZ with IC₅₀ values of 34.2 μM and 38.1 μM, respectively. In addition, these two compounds have a strong anti-tuberculosis activity against *M. tuberculosis* H37Ra with MIC values of 0.25 μg mL⁻¹ and 2 μg mL⁻¹ [109]. As a continued study, Reynolds and co-workers further investigated the biological activities by using pyridopyrazine and pyrimidothiazine analogs [110]. The results showed that most of the compounds possess strong antimicrobial activity. However, they exhibited high cytotoxicity on mammalian cells. The SAR study revealed that pyridopyrazine derivatives possessing the heteroaromatic substituents at the C6 and C7 positions were more potent than the other compounds. In addition, a carbamate group substituted at the C2 position was critical for having better biological activity. Most of the compounds exhibit specific inhibitory effects on FtsZ polymerization without any effect on tubulin. Compounds 34 and 36 exhibited excellent antibacterial activity against *M. tuberculosis* H37Rv with MIC values smaller than 0.19 μM and a moderate inhibitory activity against *M. tuberculosis* FtsZ polymerization (IC₅₀ around 34 μM). Moreover, 34 shows positive effects on the acute TB infectious mice model. Pyrimidothiazine analogs of 35 were also synthesized and evaluated but were less potent than 35 [110].

8.3.2.7 Quinazoline derivatives

Zantrin Z3 (37 in Figure 6), a quinazoline derivative, was reported as a GTPase inhibitor of FtsZ with an IC₅₀ of 24 μM by Margalit and co-workers through a high throughput screening assay [111]. This compound was reported to perturb Z-ring assembly in *E. coli* cells and cause lethality to a variety of bacteria in broth cultures [111]. To develop reliable and valuable new FtsZ inhibitors, Shaw and co-workers preformed a broad biochemical cross-comparison of many known FtsZ inhibitors for the selection of reliable
molecular scaffolds for further structural advancement. Modification of Zantrin Z3 gave 38 (in Figure 6), a compound with an IC$_{50}$ value of 12 μM for the GTPase inhibition of FtsZ [112]. As a continued study, several quinazoline analogs were synthesized and examined for their biological activity. The SAR study discovered that benzo[g]quinazoline of Zantrin Z3 could be substituted by a smaller quinazoline while the 4-chlorostyryl moiety was critical for inhibitory effect on GTPase activity of FtsZ. Among these analogs, 39 (in Figure 6) having an ammonium salt side chain shows the best potency against GTPase of FtsZ with an IC$_{50}$ of 9 μM [113].

![Figure 6. Structures of benzopyridines, rhodanine derivatives, pyridopyrazine and pyrimidothiazine analogs, and quinazoline derivatives](image)

### 8.3.2.8 Taxane derivatives

Modification of taxane-derived molecules was also conducted in order to target FtsZ more favorably than tubulin. The main scaffold of taxane polycyclic core was kept unchanged in this study. SB-RA-2001 (40 in Figure 7) shows differences from paclitaxel (41 in Figure 7) mainly in two parts: (i) the alcohol...
at C-10 without the acetyl group; (ii) an unsaturated ester replaces the \( \alpha \)-hydroxy-\( \beta \)-amido ester at C-13 [114]. These structural modifications resulted in better inhibition of \( B.\ subtilis \) FtsZ activity and showed antibacterial activity against both \( B.\ subtilis \) and \( M.\ smegmatis \). However, it is highly cytotoxic towards mammalian cells. As a continued study, Ojima et al. disclosed that the conjugated ester group when it is coupled with a ring-opened core (TRA 10a (42 in Figure 7) or 10b (43 in Figure 7)) can enhance the potency of taxane-derived small molecule targeting at \( M.\ tuberculosis \) FtsZ. Moreover, these compounds are clearly much less cytotoxic than paclitaxel (200–1,000 times less toxic) and 40 against mammalian cells [115].

8.3.2.9 Benzimidazole derivatives

Albendazole and thiabendazole are known tubulin inhibitors [116]. Previous reports revealed that these two benzimidazole derivatives inhibits septation of \( E.\ coli \) and \( M.\ tuberculosis \) cells leading to cell elongation and death [116,117]. Through structural analysis of these compounds, Ojima and co-workers synthesized a number of new small organic molecules with the benzimidazole scaffold (Figure 7) and evaluated their antimicrobial activity against \( M.\ tuberculosis \) H37Rv. Among these compounds, 44 and 45 (Figure 7) were found to display MIC values lower that 1 \( \mu \)g mL\(^{-1}\) without any significant toxicity (IC\(_{50}\) > 200 \( \mu \)M) against Vero cells [118]. Further investigation revealed that these compounds also showed similar potency against the drug-resistant \( M.\ tuberculosis \) strains. They showed a dose-dependent inhibition of FtsZ polymerization and exhibited a very strong improvement on the GTPase activity. TEM and SEM analyses of \( M.\ tuberculosis \) FtsZ and cells revealed that these compounds can perturb the size and thickness of FtsZ polymers and induce cell filamentation.

Ojima and co-workers further conducted a structural modification based on the reported lead compounds 44 and 45 by optimizing the nitrogen substituents at the 5- and 6-position while keeping the cyclohexyl group at the 2-position unchanged. They thus discovered several new compounds having MIC values that are below 1 \( \mu \)g mL\(^{-1}\) against \( M.\ tuberculosis \) H37Rv. Among these compounds, 46 and 47 (Figure 7) were reported to effectively inhibit clinical isolates of \( M.\ tuberculosis \) possessing different resistance profiles, with MIC values ranged from 0.06 to 0.16 \( \mu \)g mL\(^{-1}\). Moreover, light scattering assay and TEM analyses indicated that these compounds obviously inhibited polymerization of \( M.\ tuberculosis \) FtsZ [119]. From all these results, it was suggested that the antimicrobial activity of these trisubstituted benzimidazoles can be attributed to their interaction with \( M.\ tuberculosis \) FtsZ. To evaluate the drug-like properties of these compounds, they studied the stability and \textit{in vitro} susceptibility of the representative compound 48 (Figure 7). In the study, 48 showed outstanding stability in liver microsomes and human plasma. In addition, 48 was efficacious in the tuberculosis (TB) infectious animal model.
The results suggested that these tri-substituted benzimidazoles can be further optimized for development of novel antibacterials targeting FtsZ.

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8.3.3. Small peptides

A cathelin-related antimicrobial peptide (CRAMP) of 37 amino acid residues was found in multicellular organisms and reported to provide innate immunity to fight against microbes. CRAMP induces elongation of bacterial cells, indicating that it inhibits bacterial cytokinesis [121]. Recently, Ray et al. reported that a truncated version of CRAMP (GEKLKKIGQKINFFQKL, 16–33) exhibited antibacterial activity and FtsZ inhibitory effects [122]. This peptide can totally inhibit the growth of *B. subtilis* and *E. coli* at concentration of 20 μM and 50 μM, respectively. Light scattering assay of FtsZ with this peptide showed a dose-dependent inhibitory effect on FtsZ polymerization. By contrast, CRAMP does not affect tubulin polymerization. In addition, this
peptide also decreases GTPase activity of FtsZ, and inhibits cell division and Z-ring formation of *B. subtilis*, leading to cell elongation.

Using computational alanine scanning (CAS), Pieraccini *et al.* identified the hot spot on the protein-protein interface between FtsZ subunits and used the derived interactions map to select an octapeptide corresponding to an FtsZ subsequence, which could interfere with FtsZ self-assembly. This peptide was then modified through two different schemes of cyclisation to constrain its helical geometry. These cyclic peptides are notably successful in inhibiting the rapid initial polymer formation and causing de-polymerization in response to the reduction in GTP in the initial steady phase of polymerization. These peptides interfere with both the assembly and the enzyme activity of *E. coli* FtsZ [123].

### 8.3.4. Summary of FtsZ inhibitors

A summary of the above-mentioned FtsZ inhibitors, bacterial strains investigated, tentative binding sites, and their mode of action is given in Table 1. Since only PC 190723 and its analogs have co-crystals with FtsZ protein, more information on the structural biology of inhibitors with the FtsZ protein is needed in order to promote structure-based rational design of new small molecules. Nevertheless, the putative binding sites have been discussed with the potent compounds and their possible mechanisms of action have also been proposed based on various biochemical studies.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Organism studied</th>
<th>MIC (μg mL⁻¹)</th>
<th>Tentative Binding site</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Berberine [75]</td>
<td><em>B. subtilis</em>; <em>E. coli</em></td>
<td>100 &gt;400</td>
<td>Hydrophobic core of GTP binding site</td>
<td>Inhibits GTPase activity of <em>E. coli</em> FtsZ</td>
</tr>
<tr>
<td>Berberine derivative (2) [76]</td>
<td>MRSA; VRE</td>
<td>2 4</td>
<td>Interdomain cleft</td>
<td>Inhibits GTPase activity and polymerization of <em>S. aureus</em> FtsZ</td>
</tr>
<tr>
<td>Cinnamaldehyde [77]</td>
<td><em>B. subtilis</em>; <em>E. coli</em></td>
<td>4 1000</td>
<td>T7 loop</td>
<td>Inhibits GTPase activity and polymerization of <em>E. coli</em> FtsZ</td>
</tr>
<tr>
<td>Cinnamaldehyde derivative (4) [78]</td>
<td>MRSA</td>
<td>4</td>
<td>T7 loop</td>
<td>Inhibits GTPase activity and polymerization of <em>S. aureus</em> FtsZ</td>
</tr>
<tr>
<td>Compound</td>
<td>Organism studied</td>
<td>MIC (μg mL⁻¹)</td>
<td>Tentative Binding site</td>
<td>Mode of action</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Chrysophaeatin A [79]</td>
<td>MRSA; VRE</td>
<td>1.5; 2.9</td>
<td>GTP binding site</td>
<td>Inhibits GTPase activity and polymerization of E. coli FtsZ</td>
</tr>
<tr>
<td>Curcumin [82]</td>
<td>B. subtilis; E. coli</td>
<td>37; &gt;200</td>
<td>GTP binding site</td>
<td>Increases GTPase activity and destabilizes FtsZ polymerization</td>
</tr>
<tr>
<td>Viriditoxin [83]</td>
<td>S. aureus; E. faecium</td>
<td>4–8; 2–16</td>
<td>Unknown</td>
<td>Inhibits GTPase activity and polymerization of E. coli FtsZ</td>
</tr>
<tr>
<td>Coumarins [84]</td>
<td>B. subtilis; M. tuberculosis</td>
<td>100; &gt;100</td>
<td>T7 loop</td>
<td>Inhibits GTPase activity and polymerization of FtsZ</td>
</tr>
<tr>
<td>Plumbagin [85]</td>
<td>B. subtilis; E. coli</td>
<td>48; &gt;100</td>
<td>T7 loop</td>
<td>Inhibits GTPase activity and polymerization of B. subtilis FtsZ</td>
</tr>
<tr>
<td>Dichamanetin and 14 [89]</td>
<td>S. aureus</td>
<td>0.8; 1.16</td>
<td>Unknown</td>
<td>Inhibits GTPase activity and of E. coli FtsZ</td>
</tr>
<tr>
<td>PC190723 [25,91]</td>
<td>S. aureus; MRSA</td>
<td>1</td>
<td>Interdomain cleft between C-terminal and H7 Helix</td>
<td>Stabilizes FtsZ polymerization</td>
</tr>
<tr>
<td>Arene-diol digallate (26) [98]</td>
<td>MRSA</td>
<td>7 μM</td>
<td>GTP binding site</td>
<td>Inhibits GTPase activity</td>
</tr>
<tr>
<td>Pyrimidine-quinuclidine derivative (27) [100]</td>
<td>S. aureus; E. coli</td>
<td>12; 24</td>
<td>GTP binding site</td>
<td>Inhibits GTPase activity and polymerization of S. aureus FtsZ</td>
</tr>
<tr>
<td>Benzopyridine derivative (30) [103]</td>
<td>MRSA; VRE</td>
<td>0.5; 2</td>
<td>Unknown</td>
<td>Increases FtsZ polymerization</td>
</tr>
<tr>
<td>Rhodanine derivatives [107,108]</td>
<td>B. subtilis;</td>
<td>1–2</td>
<td>T7 loop</td>
<td>Increase FtsZ polymerization</td>
</tr>
</tbody>
</table>
### Table 1. (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Organism studied</th>
<th>MIC (μg mL⁻¹)</th>
<th>Tentative Binding site</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridopyrazine and pyrimidothiazine analogs [109]</td>
<td><em>M. tuberculosis</em></td>
<td>0.25–2</td>
<td>Unknown</td>
<td>Inhibit the polymerization of <em>M. tuberculosis</em> FtsZ</td>
</tr>
<tr>
<td>Zantrin Z3 [111]</td>
<td><em>S. aureus; E. coli</em></td>
<td>5</td>
<td>Unknown</td>
<td>Stabilizes FtsZ polymerization</td>
</tr>
<tr>
<td>Taxane derivative [115]</td>
<td><em>M. tuberculosis</em></td>
<td>2.5–10 μM</td>
<td>C-terminal region around T7 loop</td>
<td>Increases FtsZ polymerization</td>
</tr>
<tr>
<td>Benzimidazole derivatives [118,120]</td>
<td><em>M. tuberculosis</em></td>
<td>0.06–0.16</td>
<td>Unknown</td>
<td>Increase GTPase activity, and inhibit FtsZ polymerization</td>
</tr>
<tr>
<td>CRAMP [122]</td>
<td><em>B. subtilis; E. coli</em></td>
<td>20 μM; 50 μM</td>
<td>Near T7 loop</td>
<td>Disrupts FtsZ-FtsZ interaction</td>
</tr>
</tbody>
</table>

### 8.4. CONCLUSIONS AND FUTURE TRENDS

Infections caused by drug-resistant bacteria, in particular multidrug resistant strains, are a great pending threat to human health. However, most of the clinically used drugs are still limited to classical antibacterial agents such as those that target at the penicillin binding protein. Thus, there is an urgent need to develop new generation of antibacterial agents that have a different mechanism of action comparing to the existing ones.

In the last two decades, FtsZ was considered as a promising new target for development of next-generation antibacterial agents. FtsZ is a highly conserved protein among bacteria and it plays an essential role in bacterial cell division. The inhibition of FtsZ activity causes the inhibition of bacterial cell division and disruption of septum formation, leading to cell death. New types of FtsZ inhibitors therefore have been investigated actively for their antibacterial activity against various bacterial strains. Moreover, extensive investigations have been conducted on the structures and functions of the FtsZ protein based on its structural and molecular biology. The findings can be translated into structure-based drug discovery through exploiting computational biology tools.

As discussed above, various FtsZ inhibitors are potentially leads for further structural modification and biochemical investigation. The benzamide
derivative PC190723 [91] shows a strong antibacterial activity against various
*S. aureus* strains. Because of its poor drug-like properties, some powerful
prodrugs were developed based on the PC190723, and these compounds are
efficacious in the infectious animal model [96,124]. On the other hand, natural
products are regarded as abundant sources for the drug discovery. For
berberine [75], identified as FtsZ inhibitor, its structural modification has
produced several compounds with potent antibacterial activity against both
Gram-positive and Gram-negative strains. In addition, these derivatives were
found to possess a better inhibitory effect on the FtsZ activity compared with
their parent compound [76,104]. Moreover, the development of target
validation assays and efficient screening methods would also accelerate the
discovery of FtsZ targeting compounds from natural source.

Although some FtsZ inhibitors have been known to possess potent
antibacterial activity, no one has emerged for clinical trial yet. The discovery of
FtsZ targeting compounds seems to be trapped in the very early stages. One
possible reason is that the structural information on FtsZ co-crystals with
inhibitors is still insufficient. Although more than 30 crystal structures of FtsZ
have been collected in the Protein Data Bank, around 30 % of them are from *S.
aureus*, and only PC 190723 and its analogs have the co-complex with *S. aureus*
FtsZ [25,125], which provides limited information on how FtsZ inhibitors
interact with FtsZ and affect the FtsZ function. PC190723 locates in the
interdomain cleft between C-terminal and H7 helix of FtsZ in a flat orientation
and performs to stabilize the FtsZ assembly by shifting the H7 helix.
Nonetheless, due to the lack of crystallographic data, it is difficult to develop
better inhibitors based on current structural biology results. In fact, additional
interaction information about FtsZ binding to its inhibitors could provide
important insights into molecular design of inhibitors.

In conclusion, FtsZ has been considered as an excellent drug target for
development of antibacterial agents and extensive efforts have been
contributed on searching for potent FtsZ inhibitors as the next-generation
antibiotics. Accordingly, we are optimistic that that a handful of clinical
candidates could be developed in the coming future.

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THE ONLY WAY TO FILL THE GAP: COMBINATIONS OF REPURPOSED DRUGS AGAINST ANTIBIOTIC RESISTANCE AND LETHAL SEPSIS

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Chapter 9

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9.1. INTRODUCTION

Discovery of new antibiotics that are effective against Gram-negative bacteria has proven very difficult in recent decades. Even if increased funding reinvigorates efforts, it is far from certain that sufficient truly novel chemical classes will be discovered. In fact, according to Coates et al the chance that sufficient new classes of antibiotics will be discovered is remote [1].

If it is unlikely the gap will be filled in time to impact significantly on the increase in deaths predicted over the next two decades, then it is essential that we make better use of drugs currently available to fill the gap – not only antibiotics alone and in combinations, but also other drugs that could be used to protect the host against the foreign invader. For certain antibiotics, we may be able to repurpose other classes of drug to break resistance and rescue the antibiotic. Also, often it is not bacteria that kill, it is our body’s reaction to the bacteria. Septic shock followed by organ damage and death is caused by precipitous fall in blood pressure, blood coagulation, and by dysregulated response of the immune system to infection. Treatment of bacterial infection and sepsis is currently heavily focused on choice of which antibiotics to use: less attention is paid to modulating the inappropriate host response or tackling the cause of the fall in blood pressure, and actions to protect organs at risk are inadequate. New drug discovery efforts against sepsis have a poor record [2], but there is major opportunity to introduce rapidly new treatments by repurposing drugs already available for clinical use. This could ‘fill the gap’ by reducing use of antibiotics, slowing development of resistance, and save many lives.

Five very practical repurposing approaches to save lives are summarised in this chapter.

The first opportunity is to assess the effectiveness of beta-lactam antibiotics when combined with any two of the several resistance-breaking beta-lactamase inhibitors or dehydropeptidase inhibitors available for use. This option has been exploited only in a single combination so far, which leaves ample scope for introduction of new triple combinations (antibiotic plus two non-antibiotic resistance breakers) to break resistance against our most precious beta-lactam antibiotics.

The second opportunity is a rigorous investigation of combinations of two or more antibiotics, particularly by including those that modulate host defense and protect organs from damage. Physicians often resort to use of combinations or to sequential antibiotics, yet the scientific evidence for their use is poor. Again, only a single example of this approach being exploited exists today: only one combination of two antibiotics effective against lethal Gram-negative bacteria has been developed and approved by regulatory authorities. This represents a massive gap that could be filled by repurposing current
antibiotics in rigorously-proven combinations to extend their usefulness against resistant bacteria.

The third opportunity is to explore greater use of bacteriostatic antibiotics either alone or in combinations. This could side-step the growing issue of bacteria resistant to carbapenems. It might also help to reduce tissue damage in severe infections that lead to septic shock by not releasing bacterial components that can exacerbate inflammation and result in damage to vasculature and many internal organs. This option becomes increasingly practical if organ-protecting strategies acting via host-mechanisms are also implemented. This might be our only practical chance of beating the growing threat from carbapenem-resistant bacteria.

The fourth opportunity is to repurpose non-antibiotic drugs for treatment of sepsis. Septic shock is often the ultimate outcome of infection, leading to organ damage and failure, loss of limbs, disfigurement and a high death rate. Advances in medical science in recent years are not reflected in current treatment of sepsis, and earlier clinical trials have given extremely disappointing results [2]. Understanding the inappropriate immune activation, inflammation, vascular leakage, organ damage and blood clotting that leads to these sad outcomes can point to opportunities to repurpose safe drugs to protect the infected person from damage. These repurposed non-antibiotic drugs could be used alongside antibiotics in treatment of sepsis, and very likely lead to a significant reduction in use of antibiotics.

The fifth opportunity is to extend use of macrolides and tetracyclines in combination with other antibiotics to protect patients against the consequences of bacterial infection. These drug classes have additional host-defense properties in addition to their antibiotic effects. There is considerable clinical evidence in several types of bacterial infection that their use can lead to lower death rates than would be expected. Rigorous examination of the best combinations could reinvigorate therapeutic options.
9.2. WHY DEATHS WILL INCREASE BEFORE THE ANTIBIOTIC GAP IS FILLED

9.2.1. Current actions are too little and too late

In recent years, many publications have highlighted the growing risk to humanity from the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) [3]. Resistance levels of up to 50 % have been reported in some developing countries against carbapenems, the current last line of defence [4]. A few new classes of antibiotics against Gram-positive bacteria have become available in recent decades. However, for treatment of Gram-negative infections no totally new class of antibiotic has been introduced for over forty years, only modifications of older antibiotics, against which bacteria have been able to develop resistance quite rapidly. All chemical classes of antibiotics useful against Gram-negatives were discovered in the 1930s–1960s, none since then. Figure 1 shows the date of introduction of each new class of antibiotic since the sulphonamides in the 1930s.

![Figure 1. Date of introduction of each new class of antibiotic](image)

Why is it more difficult to invent new chemical classes effective against Gram-negative than Gram-positive bacteria? Gram-positives have a single cell membrane that antibiotics must penetrate to gain access to the bacterial cell. Gram-negative bacteria contain an additional barrier, a cell wall outside the inner membrane, the two together being called the ‘envelope’. Moreover, this second barrier has very different physicochemical properties than the inner membrane, which increases the difficulty of discovering antibiotics effective
against Gram-negatives. There is significant doubt that we can deliver new antibiotics for Gram-negatives at the rate required in coming decades.

UK Chief Medical Officer Dame Sally Davies has been a global leader in highlighting the antibiotic resistance issue and in 2014 the UK government initiated a review of the seriousness of the problem. Jim O’Neill led a team that conducted an in-depth review of antibiotic resistance. The diagram below is taken from the first report from his team, issued in December 2014, titled ‘Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations’ [5]. His team was staffed mostly by economists (his own background) and Figure 2 shows the predicted loss in World GDP in coming decades due to antimicrobial resistance. The exponential loss in global wealth is also mirrored in a similar prediction of exponential loss of lives. The O’Neill review predicted that deaths from antimicrobial resistance could overtake those from cancer in the 2040s.

![Diagram showing predicted impact of AMR on World GDP](image)

**Figure 2.** Predicted impact of AMR. Increase in deaths and reduction in global GDP [5].
The 2040s are only a quarter century away which is a short time when one remembers that timelines for drug development are measured in decades and we have already failed for 40-years to invent new antibiotics effective against Gram-negative bacteria. As Coates et al. [1] stated, a big gap in the antibiotic pipeline is guaranteed. Some governments have now started to increase public funding for antibiotic research, yet many problems remain: the amount of funding available is small in comparison with that spent in other disease areas; the global skill base is weak due to previous under-investment; clinical development is challenging; and the commercial model for antibiotics remains unsolved despite much debate about the current broken model.

A recent report titled ‘Incentivising innovation in antibiotic drug discovery and development: progress, challenges and next steps’ by Simpkin et al. [6] assessed the major international, European Union, US and UK antibiotic R&D funding programmes. They found that incentive programmes are overly committed to early-stage push funding of basic science and preclinical research, while there is limited late-stage push funding of clinical development. Moreover, there are almost no pull incentives to facilitate transition of antibiotic products from early clinical phases to commercialisation, or to focus developer concentration on the highest priority antibiotics, or to attract large pharmaceutical companies to invest in the market.

9.2.2 Limitations of the current antibiotic pipeline

Also, the current pipeline of antibiotics is a major concern. According to the latest pipeline overview reported by the Pew Trust [7] updated to September 2017, 48 antibiotics with the potential to treat serious bacterial infections were identified in clinical development for the U.S. market. The number may sound encouraging but a deeper look at the compounds and their profiles reveals ‘more of the same’. Perhaps the most valuable information in the report is that only 1 of the 48 represents a potential breakthrough. Only Murepavadin was listed as representing a novel drug class, defined as a core chemical structure (scaffold) that has not previously been used systemically as an antibacterial in humans, while also hitting a novel molecular target (a target is defined as novel if the drug acts on a bacterial structure that has not previously been targeted by a systemic antibacterial in humans.) It is in phase 2 clinical trials for the narrow indication of treatment of ventilator-associated bacterial pneumonia due to *Pseudomonas aeruginosa*. Apart from this single example, all other antibiotics identified in the report do not target the ESKAPE pathogens by a new chemical class and new biochemical target. Historical data indicates that the success rate for clinical drug development of antibiotics is low. Generally, only 1 in 5 infectious disease products that enter human testing (phase 1 clinical trials) will be approved for patients. Another
concern is that even for new classes of antibiotic, resistance can develop very rapidly, within 2 years of marketing according to Bax et al. [8].

One more point should be made before closing this section. Several authors have stated that the greatest threat to humanity comes from carbapenem-resistant Enterobacteriaceae (CRE) [9]. This form of resistance is appearing in many genera including familiar pathogens such as *E. coli*, *Salmonella*, *Yersinia pestis* (plague), *Klebsiella*, and *Shigella* (dysentery). Bacteria that have acquired resistance to both carbapenemases and ESBLs can be resistant not just to carbapenems but to almost all available beta-lactams, our most widely used class of antibiotic. It is unclear whether any of the antibiotics in the pipeline address this major concern. The seriousness of the issue is illustrated in Figure 3, kindly provided by Dr John Rex.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acinetobacter baumannii</em>, carbapenem-R</td>
<td>Critical</td>
<td>Serious (MDR)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em>, carbapenem-R</td>
<td>Critical</td>
<td>Serious (MDR)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em>, carbapenem-R, 3rd-gen cephr (ESBL+)</td>
<td>Critical</td>
<td>Urgent (carbapenem-R)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em>, vancomycin-R</td>
<td>High</td>
<td>Serious (VRE)</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em>, methicillin-R, vancomycin-I/R</td>
<td>High</td>
<td>Serious (MRSA)</td>
<td>Concerning (VRSA)</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em>, clarithromycin-R</td>
<td>High</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter</em> spp., fluoroquinolone-R</td>
<td>High</td>
<td>Serious (drug-R)</td>
<td></td>
</tr>
<tr>
<td><em>Salmonella</em> spp., fluoroquinolone-R</td>
<td>High</td>
<td>Serious (drug-R)</td>
<td></td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em>, 3rd-gen cephr, fluoroquinolone-R</td>
<td>High</td>
<td>Urgent (drug-R)</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em>, penicillin-NS</td>
<td>Medium</td>
<td>Serious (drug-R)</td>
<td></td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em>, ampicillin-R</td>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella</em> spp., fluoroquinolone-R</td>
<td>Medium</td>
<td>Serious</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium difficile</em></td>
<td></td>
<td></td>
<td>Urgent</td>
</tr>
<tr>
<td><em>Candida</em> spp., fluconazole-R</td>
<td></td>
<td>Serious (Flu-R)</td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td></td>
<td>Serious (drug-R)</td>
<td></td>
</tr>
<tr>
<td>Group A <em>Streptococcus</em></td>
<td></td>
<td>Concerning (erytho-R)</td>
<td></td>
</tr>
<tr>
<td>Group B <em>Streptococcus</em></td>
<td></td>
<td>Concerning (clinda-R)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.** WHO priority pathogen list for R&D

([http://amr.solutions/blog/who-priority-pathogens-list](http://amr.solutions/blog/who-priority-pathogens-list))
9.3. REPURPOSING ANTIBIOTICS: ANTIBIOTIC COMBINATIONS TO BREAK RESISTANCE

With this serious situation, it is essential that we take urgent action to save our best antibiotics. When bacteria develop resistance to an antibiotic it may be possible to break that resistance and make the antibiotic effective again by adding a second drug molecule. There are several categories of drug molecule that might be used:

1) for beta-lactam antibiotics, use in combination with one or more additional (non-antibiotic) beta-lactamase inhibitors to prevent degradation of the antibiotic by bacteria,

2) for all antibiotics, addition of a second antibiotic,

3) combinations of bacteriostatic antibiotics to side-step MBLs and CREs.

9.3.1. Beta-lactams combined with one or two beta-lactamase inhibitors

Combinations of a beta-lactam antibiotic and a single lactamase inhibitor are receiving a lot of attention. Several combinations of this type are available on-the-market or in clinical development, as shown in Table 1.

<table>
<thead>
<tr>
<th>Antibiotic + beta-lactamase inhibitor</th>
<th>Route of administration: intravenous or oral</th>
<th>Market year or clinical development stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampicillin / sulbactam</td>
<td>iv; po</td>
<td>1987</td>
</tr>
<tr>
<td>amoxycillin / clavulanic acid</td>
<td>iv; po</td>
<td>1984</td>
</tr>
<tr>
<td>cefoperazone / sulbactam</td>
<td>iv; po</td>
<td>Not-approved</td>
</tr>
<tr>
<td>ceftazidime / avibactam</td>
<td>iv</td>
<td>2015</td>
</tr>
<tr>
<td>ceftozolane / tazobactam</td>
<td>iv</td>
<td>2015</td>
</tr>
<tr>
<td>imipenem / cilastatin (DHP)</td>
<td>iv</td>
<td>1987</td>
</tr>
<tr>
<td>piperacillin / tazobactam</td>
<td>iv</td>
<td>1993</td>
</tr>
<tr>
<td>ticarcillin /clavulanic acid</td>
<td>iv</td>
<td>1990</td>
</tr>
<tr>
<td>meropenem + vaborbactam</td>
<td>iv</td>
<td>FDA approval 2017</td>
</tr>
<tr>
<td>imipenem/ cilastatin + relebactam</td>
<td>iv</td>
<td>Clinical Phase 3</td>
</tr>
<tr>
<td>aztreonam + avibactam</td>
<td>iv</td>
<td>Clinical Phase 2</td>
</tr>
<tr>
<td>cefepime / zidebactam</td>
<td>iv</td>
<td>Clinical Phase 1</td>
</tr>
</tbody>
</table>
Those in clinical development generally have reasonable potency against *E. coli* and *K. pneumoniae* but they are much less effective (or not effective at all) against *P. aeruginosa* and *A. baumannii*. Effective treatment of these bacteria remains a major issue.

The triple combination comprising imipenem with the beta-lactamase inhibitor relebactam and the dehydropeptidase inhibitor cilastatin is the first example of the interesting idea of using two resistance breakers in combination with an antibiotic. There is considerable scope to explore this further, for instance by assessing the effectiveness of beta-lactam antibiotics combined with any two of the several beta-lactamase inhibitors available for use. Drugs with related chemical structures acting on the same target often bind that target in subtly different ways, so combining two similar-looking beta-lactamase inhibitors could be unexpectedly effective in blocking degradation of beta-lactam antibiotics.

A related idea, currently totally unexplored in the literature, is to combine two antibiotics and a beta-lactamase inhibitor. The number of possible combinations is huge, and it seems quite likely that combinations able to overcome bacterial resistance could be found.

The next section explores the status of combinations of antibiotics.

### 9.3.2. Antibiotics combined with a second or third antibiotic

Category 2 has received much less attention. Table 2 shows the available combinations of two antibiotics and Table 3 shows the single example of a combination of three antibiotics, although this is for topical use only.

<table>
<thead>
<tr>
<th>Antibiotics combined with a second antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampicillin + flucloxacillin po Not Gram-negatives Used for cellulitis due to Gram-positive <em>staphylococcus</em> and streptomyces</td>
</tr>
<tr>
<td>bacitracin/polymyxin B topical Eye infections The only systemic acting double combination against Gram-negatives</td>
</tr>
<tr>
<td>co-trimoxazole oral The only systemic acting double combination against Gram-negatives (trimethoprim+sulfamethoxazole)</td>
</tr>
<tr>
<td>phenoxymethylpenicillin/pipacycline oral, im Weak against Gram-negatives Salt formed between a tetracycline and a penicillin (Penimepcycline)</td>
</tr>
<tr>
<td>quinupristin/dalfopristin (Synercid) iv Both are pristinamycin analogues Used against <em>staphylococci</em> and vancomycin-resistant <em>Enterococcus faecium</em></td>
</tr>
</tbody>
</table>
The only way to fill the gap: combinations of repurposed drugs against...

<table>
<thead>
<tr>
<th>Table 2. (continued)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mikamycin B / streptogramin A (pristinamycin)</td>
</tr>
<tr>
<td>virginiamycin / pristinamycin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 3. Antibiotic combined with a second and third antibiotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>neomycin/polymyxin B/bacitracin</td>
</tr>
</tbody>
</table>

Tables 2 and 3 are notable in several respects. Despite the large numbers of antibiotics available (over 100) and the even-larger number of possible combinations (many thousands), there are few entries in the table. Moreover, only one entry has efficacy against Gram-negative bacteria. Co-trimoxazole became available in 1974, but no other combination of two antibiotics effective against Gram-negative bacteria has become available since then.

In severe infections, physicians often prescribe two or more antibiotics to patients either together or in sequence, yet most of those combinations have not been rigorously proven to be effective in human clinical trials nor approved by regulatory authorities. This use of unproven combinations may be contributing to the widespread development of resistance to antibiotics.

In addition, the lack of available proven combinations represents a massive gap in development of antibacterial treatments. If more combinations could be developed it could help address the pipeline gap. However, the Pew Trust report of antibiotics being developed [7] contained no combinations of two or more antibiotics.

Combinations of antibiotic plus non-antibiotic has been the subject of considerable academic research and publications yet none of these combinations are registered for use and commercially available today. Companies such as UK-based Helperby Therapeutics have products in early-to-mid stage clinical development [10] though it is unclear whether these will achieve clinical success for the most serious Gram-negative infections.

Combinations of already approved drugs can be simpler to develop than a new chemical entity. Many factors which are a risk in development of a totally new drug are substantially derisked when old drugs are repurposed: for each individual drug, pharmacokinetics and distribution in the body are known; the safety profile is known; the dose required for efficacy is known etc. However, when two drugs are used in combination, drug-drug interactions need investigating to assess whether any of these parameters change. In a positive sense, if a synergist effect is achieved between two drugs, this can enable us to reduce drug levels for greater safety, or to maintain drug levels to overcome
bacterial resistance. Ideally, we would want to avoid using higher doses than those currently approved for use.

An advantage of finding unexpected synergy between two known antibiotics would be the potential to obtain a stronger patent position. This could be important for commercial return to the manufacturer.

Combinations are used extensively against other infectious diseases (TB, AIDS, Hep C) to prevent and overcome resistance, yet they have been largely ignored for antibiotics. One issue though is that the number of possible combinations and required assays is huge when one considers the large number of possible antibiotics (or even non-antibiotics), the large number of pathogenic bacteria, and the large number of resistant strains that develop for each type of bacterium. So, it is fortunate that new informatics methods are being developed based on Machine Learning to predict combinations that could be effective against specific resistant strains [11]. Also, testing protocols have been proposed which reduce the number of assays when compared with the traditional chequerboard method, which can be very expensive and logistically prohibitive for higher-order interactions [12-15].

In summary, there is a clear unmet need to develop antibiotic combinations to salvage our best antibiotics and preserve their usefulness during the decades before new chemical classes of antibiotic become available. Particularly, there is terrific opportunity for rigorous development of combinations of two or more antibiotics that are effective against resistant species of Gram-negative bacteria. These alone could go a long way towards filling the pipeline gap and reducing the extraordinary increase in death rates predicted by the O’Neill Review.

9.3.3. Combinations of bacteriostatic antibiotics to side-step MBLs and CREs and reduce organ damage in septic shock

Antibiotics are broadly classified into those that kill bacteria (‘cidal) and those that reduce growth with reliance on the immune system to clear them (‘static). There may be an assumption that bactericidal antibiotics are more effective in curing patients, however this has not been rigorously tested and it is possibly inaccurate. Recently, Nemeth et al. conducted the first ever meta-analysis of trial data comparing ‘cidal and ‘static antibiotics. Their analysis showed no difference in outcomes for ‘cidal versus ‘static antibiotics in infections affecting lungs (pneumonia), abdomen and soft tissue [16]. There was insufficient data to assess infections of brain (meningitis) and heart (endocarditis).

The bactericidal antibiotics include aminoglycosides, beta-lactams, quinolones, glycopeptides and lipopeptides; whereas the bacteriostatic antibiotics include the tetracyclines, lincosamides, macrolides, oxazolidinones, streptogramins and sulphonamides. Bactericidal antibiotics are generally recommended for
patients with a compromised immune system (such as neutropenia) yet the analysis by Nemeth et al. did not support the assumption of inferior outcomes if bacteriostatics were used. In addition, the assumption that relapse rates will be higher with bacteriostatic antibiotics was challenged by their analysis.

The emerging crisis of CRE’s and bacteria expressing ESBLs and MBLs seems unlikely to be contained by invention of sufficient classes of beta-lactam antibiotics in coming decades [11]. Alternative approaches need testing urgently. If host-mediated mechanisms can be exploited to boost host defence and protect organs, the improved therapeutic options might include replacement of bactericidal antibiotics such as beta-lactams, aminoglycosides, and quinolones with bacteriostatic antibiotics such as macrolides, tetracyclines and sulphonamides. This requires testing.

9.4. REPURPOSING NON-ANTIBIOTIC DRUGS TO PREVENT ORGAN DAMAGE AND DEATH FROM SEPSIS

Bacterial infection can cause sepsis then septic shock (development of severe hypotension and blood coagulation leading to multi-organ failure) which often is the ultimate killer. The sequalae are shown in Figure 4.

Once septic shock sets in, death rates are high. The global burden of sepsis is estimated to be 15–19 million cases annually, with a mortality rate of 20–50 % in developed countries and approximately 60 % in low-income countries [17]. The probability of death increases by approximately 10 % for each hour the condition is unrecognised or treated ineffectively.

Sepsis is a complex condition characterized by the simultaneous activation of inflammation and coagulation in response to bacterial infection. These events manifest as systemic inflammatory response syndrome (SIRS) and other symptoms through the release of proinflammatory cytokines, procoagulants, and adhesion molecules from immune cells and/or damaged endothelium. Hypoperfusion due to low blood pressure leads to organ dysfunction and failure, and to blood clotting and extensive formation of thrombi. Dysfunction of respiratory and cardiovascular systems is common, and brain and kidneys are also often affected. Systemic thrombocytopenia leads to disseminated intravascular coagulation (DIC) and survivors often have organ damage or loss of limbs, disfigurement, impaired neurocognitive function, mood disorders, and a poor quality of life. Clinical studies have shown that up to 60 % of sepsis survivors exhibit permanent cognitive deficits and memory loss [18]. Survivors also have an increased risk of death in the following months and years [19].
Figure 4. Bacterial invasion leading to septic shock

Treatment of sepsis is currently limited to use of antibiotics plus supportive measures such as use of vasopressive drugs and non-specific anti-inflammatory steroids [20]. The proximal host-response factors that lead to the severe hypotension, coagulation and inflammation are not treated directly, only the symptomology.

Severe sepsis occurs from both community-acquired and nosocomial (hospital acquired) infections. In about one-half of all cases the primary site of infection is the lung (pneumonia). Other major sites are the abdomen and the urinary tract [21-23]. The ESKAPE pathogens are the most common causes: Gram-positives *Staphylococcus aureus* and *Streptococcus pneumoniae*, and Gram-negatives *Escherichia coli*, *Klebsiella species*, and *Pseudomonas aeruginosa*.

A 2009 study involving 14,000 intensive care unit (ICU) patients in 75 countries, indicated that Gram-negative bacteria were isolated in 62% of patients with severe sepsis who had positive cultures, Gram-positive bacteria in 47%, and fungi in 19%. The figures suggest multiple infections can be present [23].

Since current treatments do not target the mechanistic cause of sepsis syndrome, this likely makes antibiotic treatment much less effective than it could be if more-specific interventions were used. Also, this likely leads to generation of more antibiotic resistance than would otherwise occur if the causes of septic shock could be rectified more directly and more rapidly.

Angus and Poll [24] have provided an excellent review of pathways involved in sepsis, and D’Elia et al. [25] have described the ‘cytokine storm’ that is characteristic of septic shock. The key cellular and biochemical changes driven
by presence of bacteria in the blood stream, lungs or peritoneal cavity include the activation of macrophages and monocytes, with tumor necrosis factor-alpha (TNF-\(\alpha\)), interleukin-1\(\beta\) (IL-1\(\beta\)), interleukin-6 (IL-6) and interleukin-8 (IL-8) particularly involved in the development of septic shock. However, the precipitous drop in blood pressure might be cause by another factor, reviewed in the next section.

9.4.1. Vitamin C, thiamine and hydrocortisone triple therapy

Clinical evidence for use of high dose vitamin C alongside standard therapy for treatment of sepsis has generated considerable interest and controversy recently. An excellent 2018 review of the pharmacological actions of vitamin C and clinical data for its use in sepsis patients has been provided by Teng et al. [26]. At the present time, based on studies in animals and a small number of patient outcomes, it appears to be a promising therapy though definitive double blind clinical trials are lacking.

The underlying hypothesis is as follows. Vitamin C is an essential cofactor in the biochemical synthesis of several vascular pressor agents that maintain blood pressure, including vasopressin and the catecholamines adrenaline (epinephrine) and dopamine. Lethal drop in blood pressure is the defining characteristic of bacterial septic shock and low levels of both vitamin C and the pressor agents have been observed in critically-ill patients. Levels of vasopressin and vitamin C deplete as a patient progresses into hypotension and shock [27-30]. It has been known for over 2 decades that the low plasma ascorbic acid concentrations in septic patients correlate inversely with the incidence of multiple organ failure and correlate with survival rates [31]. The low levels of vitamin C in septic patients has been attributed to several factors including its destruction by free radicals [32].

Vitamin C acts as co-factor for the enzyme peptidylglycine alpha-amidating monooxygenase (PAM), which is required for the synthesis of vasopressin [33]. Vitamin C is also a cofactor in two steps in the synthesis of dopamine and adrenaline. Figure 3 from Zipursky et al. [34] describes the biochemical pathway. Vitamin C is involved in the rate-limiting step of synthesis of L-DOPA, the precursor of dopamine and it is also required as a cofactor for the enzyme dopamine beta hydroxylase [33] which converts dopamine to adrenaline (epinephrine). Adrenaline is a critical vasopressor for septic patients through its action on both alpha and beta-adrenergic receptors to maintain vascular tone and increase cardiac output.
Figure 3. Vitamin C (ascorbic acid) is required to synthesize catecholamines\textsuperscript{34}

As an additional factor, vitamin C binds to and modulates function of both alpha-adrenergic and beta-adrenergic receptors and enhancing their activation by adrenaline / epinephrine [35].

Current treatment of sepsis [36] includes exogenous administration of noradrenaline (norepinephrine), adrenaline and vasopressin as necessary to maintain blood pressure, and several authors have argued that intervention to raise plasma vitamin C levels in septic shock patients might provide an effective replacement [33].
Vitamin C also has effects on the immune system. It is found in high concentration in leukocytes [37] and it amplifies in oxidative neutrophilic killing of bacteria by leukocytes [38]. Deficiency in vitamin C was associated with delayed killing of bacteria by natural killer (NK) cells and suppressed cytotoxic T cell activity [39] It inhibits tumour necrosis factor (TNF)-induced production of intercellular adhesion molecules (ICAMs), which decreases leukocyte stickiness and improves microcirculatory flow [39,40].

Additionally, vitamin C has been shown to support immune function by inhibiting apoptosis and protecting endothelial progenitor cells [41,42]. High dose vitamin C can decrease NF-kB leading to modulation of an over-active immune response [43].

In 2001, an early indication of the potential efficacy of high dose vitamin C in treatment of septic shock was provided by Armour et al. [44]. They showed that in a cecal ligation and perforation (CLP) rat model, plasma vitamin C levels plummeted by 50 % with a concomitant increase of 1,000 % in urine ascorbate concentration. This was accompanied by a 20 % decrease in mean arterial pressure, and a 30 % decrease in the density of perfused capillaries. These changes could be reversed with a bolus of intravenous ascorbate (7.6 mg/100 g body weight) administered immediately after the CLP procedure. They also showed that these high plasma levels of ascorbate might have direct killing effects on bacteria: in vitro experiments showed that ascorbate (100 μM) inhibited replication of bacteria and prevented hydrogen peroxide injury to cultured microvascular endothelial cells. The authors concluded that their studies supported a potential beneficial effect of ascorbate in patients with septic syndrome.

A series of papers from Wu et al. linked the action of ascorbate to inhibition of iNOS expression followed by reduction in peroxynitrite formation and its negative effects on endothelial function [45-48]. Working with one of the ESKAPE pathogens, in 2006 Gaut et al. reported that mice with vitamin C deficiency had a 3-fold increase in rate of death from *Klebsiella pneumoniae* infection versus those supplemented with ascorbate [49].

More recently in 2011, in a mouse model vitamin C was shown to attenuate lipopolysaccharide (LPS) mediated lung injury during sepsis [50].

Then in 2012, Zhou et al. showed in a CLP model that high dose vitamin C given 3 hours after CLP prevented an increase in vascular permeability and leakage by inhibiting excessive production of NO, superoxide and peroxynitrite [51].

One potential issue with animal studies using vitamin C is that most animals, including rats and mice, can synthesise their own vitamin C, whereas humans lack that capability – our vitamin C must come from exogenous sources such as food or supplements. Fisher et al. used a mouse model in which the genes governing vitamin C synthesis had been deleted, to more closely resemble the human situation [52]. They then induced sepsis in these mice by
intraperitoneal infusion of a fecal stem solution. This produced injury to lungs, kidneys and liver in vitamin C deficient mouse, but the mice dosed with exogenous vitamin C had reduced multiple organ dysfunction. Moreover, the gene-deleted mice developed significant abnormalities in the coagulation system and circulating blood cells. These were attenuated by vitamin C infusion. The protective action of vitamin C on vascular endothelium has also received attention [53,54].

In parallel with these in vitro and animal studies, evidence has accumulated in human studies to show a protective effect of high dose vitamin C against severe organ damage and bacterial infection. In 2002 Nathens et al. enrolled 595 patients, mostly victims of trauma [55]. They showed that multiple organ failure was significantly less likely to occur in patients receiving a combination of ascorbic acid and alpha-tocopherol (vitamin E) than in patients receiving standard care, with a relative risk of 0.43. Patients randomized to antioxidant supplementation also had a shorter duration of mechanical ventilation and length of ICU stay. Crimi et al. reported similar findings [56].

The animal studies with vitamin C in sepsis models aroused interest in human clinical trials. Wilson (2013) [57] and Carr et al. (2015) [58] summarised the rationale for testing vitamin C in a randomised clinical trial. In 2014, Fowler et al. reported a Phase 1 safety study with high dose parenteral vitamin C in 24 patients with severe sepsis [59]. In the septic patients, plasma ascorbic acid levels at entry were about one-third normal levels (17.9 ± 2.4 μM compared to a normal range of 50–70 μM). Infusion of high dose vitamin C (up to 200 mg kg⁻¹/24 h) raised plasma levels rapidly without any significant adverse events. Patients receiving the vitamin C showed rapid improvement in Sepsis-related Organ Failure Assessment (SOFA) symptomatic score. They also had significant reduction in the proinflammatory biomarkers C-reactive protein and procalcitonin. Moreover, in the treated patients thrombomodulin exhibited no significant rise, suggesting attenuation of vascular endothelial injury.

In 2016 Zabet et al. evaluated the effect of high-dose ascorbic acid on vasopressor drug requirement in critically ill patients with septic shock [60]. In 14 treated patients compared to 14 untreated controls, 28-day mortality was significantly lower in the ascorbic acid than the placebo group (14.28 % vs. 64.28 %, respectively; P = 0.009).

Then in 2017, Marik et al. reported results of a phase 2 trial in patients with severe sepsis [61]. 47 septic controls receiving normal care, including antibiotics, norepinephrine and hydrocortisone, were retrospectively compared with 47 septic patients additionally treated with intravenous vitamin C, hydrocortisone, and thiamine (the thiamine was included to reduce concerns about oxalic acid formation in kidneys from the high vitamin C doses used, and because thiamine deficiency is common in sepsis, occurring in
approximately one-third of patients and associated with increased mortality [62].

During the treatment period patients with severe sepsis or septic shock and a procalcitonin level >2 ng mL\(^{-1}\) were treated with intravenous vitamin C (1.5 g every 6 h for 4 days or until ICU discharge), hydrocortisone (50 mg every 6 h for 7 days or until ICU discharge followed by a taper over 3 days), as well as intravenous thiamine (200 mg every 12 h for 4 days or until ICU discharge). The vitamin C was administered as an infusion over 30–60 min and mixed in a 100 mL solution of either dextrose 5 % in water or normal saline. Mortality was 8.5 % (4 of 47) in the treatment group compared with 40.4 % (19 of 47) in the control group (P < 0.001). No patient in the treated group developed progressive organ failure, allowing all patients in the treatment group to be weaned off vasopressors at a mean of time of 18.3+/−9.8 h after starting treatment with the vitamin C protocol.

This was not a blinded random clinical trial and so additional studies are required to confirm these preliminary findings. The publication of these results has sparked intensive debate around the study design, which the authors accept needs upgrading in later studies. To strengthen the case, Marik has more recently stated:

“We have now treated over 150 patients with severe sepsis and septic shock. We have had only one patient die from sepsis, this being a complex surgical case who died in the immediate post-operative period. While a few of the treated patients have died, none died from progressive organ failure related to sepsis. All these patients were weaned off pressors/mechanical ventilation and died from their underlying disease. There can be no question of doubt that we have changed the natural history and disease progression of patients with sepsis... patients with sepsis simply just don’t develop progressive organ failure.” [63]

The studies from Marik et al. evaluated the synergistic administration of vitamin C with hydrocortisone and thiamine, and did not examine vitamin C monotherapy. Although the study is limited by its open label and single centered design, with small sample size, it did produce a statistically significant result with well-matched controls [64].

When viewed alongside the earlier preclinical data and the clinical studies from Fowler [59] in 2014 and Zabet [60] in 2016, and Marik's own updated report of treatment of a larger number of patients [63], the impression is strengthened that a breakthrough might have been achieved. However, we need at least two random clinical trials to confirm the effect of treatment with high dose vitamin C. We also need to study the relative contributions of the various other agents used, and the best dosage regimes.

Even if eventually proven useful, it seems unlikely that vitamin C will provide full efficacy when larger numbers of patients are studied in other trial centres, and the following sections of this paper review some other readily available
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drugs that could also be considered for use as adjuncts to antibiotic treatment to reduce death rates from sepsis.

One other factor should be considered. If high dose intravenous vitamin C does prove to be a life-saver in septic patients it may be worthwhile to rethink the accompanying antibiotics. For instance, would bacteriostatic agents be better than the bactericidal antibiotics which are currently used? Bactericidal drugs lead to bacterial cell lysis and release of inflammatory mediators such as LPS and bacterial DNA, which could be worsening the excessive inflammatory responses during treatment of sepsis. In addition, an over-reliance on beta-lactam antibiotics is leading to major resistance to this antibiotic class, including the growing concern about CREs mentioned earlier. As the usefulness of non-antibiotic adjunct therapies begins to change overall treatment regimes, there may be a case for revisiting other classes such as those antibiotics which also have immune-modulating actions that are discussed in a later section of this article.

9.4.2. Imatinib and abl kinase inhibitors

Vascular endothelial damage is a primary event leading to other pathological symptoms of septic shock. The vascular endothelial barrier breaks down and fluid leaks into the extravascular space. This leads to life-threatening oedema in internal organs including lung, kidney and brain, with multi-organ failure.

Several groups have reported that an FDA-approved anti-cancer drug, the Abl-related gene (ARG) kinase inhibitor imatinib, can attenuate inflammatory vascular leak. Interest in imatinib was roused initially by the serendipitous observation that the drug reversed pulmonary oedema in some patients [65]. Subsequently, imatinib has been shown to reduce vascular leak induced by a broad range of challenges including thrombin, histamine, vascular endothelial growth factor (VEGF), oxidative stress, and (importantly) LPS which is a component of Gram-negative bacteria cell wall [66]. In a murine model of sepsis imatinib treatment (6 h and 18 h after induction of sepsis) attenuated vascular leakage in the kidneys and the lungs 24 h after induction of sepsis [67]. In vivo, it completely prevented mortality in an intravenous LPS mouse model of endotoxemia and lung injury [68] and it protects against LPS-induced acute lung injury with decreased production of pro-inflammatory mediators [69].

Imatinib has proven to protect organs from damage in other murine models: it preserves the integrity of the blood-brain-barrier (BBB) and reduces inflammation by modulating the peripheral immune response [70]. These observations suggest that inhibition of Abl kinases may have therapeutic potential for decreasing the vascular permeability that underlies much of the organ damage in sepsis. Several other inhibitors of this enzyme are available
The underlying physiological mechanism of action is reasonably well established. The Abl family kinases, c-Abl (Abl1) and Abl related gene (Arg, Abl2), have well characterized roles in the dynamic regulation of the actin cytoskeleton and cell-cell and cell-matrix junctions [71,72]. Abl kinases phosphorylate several cytoskeletal proteins involved in vascular permeability.

However, imatinib is not totally selective for Abl kinase, and inhibition of other kinases may be involved in its efficacy. For example, PDGF-R kinase is a target of imatinib and other FDA approved class members. Additional studies are warranted to determine the contribution of each of these targets in view of their potential in treatment of inflammatory vascular leak syndromes including sepsis.

9.4.3. Sildenafil and phosphodiesterase-5 inhibitors

A growing body of literature describes the organ-protective effects of inhibition of the enzyme phosphodiesterase-5 (PDE5) during severe bacterial infection. This enzyme is the target of drugs developed originally for male erectile dysfunction, such as Viagra. Sildenafil (the active ingredient of Viagra) has a broad range of tissue-protective properties including many organs that are vulnerable to damage during septic shock.

Cadirci et al. in 2011 published the first study in animals that showed sildenafil to be protective when dosed post-operatively in a model of bacterial septic shock [73]. In the rat model of caecal ligation and puncture (CLP)-induced sepsis, they demonstrated that sildenafil treatment attenuates lung and kidney injury due to overproduction of oxidant activity. Histopathological analysis showed significant protection against inflammation. Biochemical analysis showed that sildenafil decreased serum level of TNF-α. The authors concluded ‘sildenafil is a highly protective agent in preventing lung and kidney damage caused by CLP-induced sepsis via maintenance of the oxidant / anti-oxidant status and decrease in the level of TNF-α.’

The same year Carvalho et al. also analysed the effects of sildenafil in the CLP model in rats pretreated with the drug [74]. They confirmed the effect on TNF-α, and also showed that sildenafil reduced levels of C-reactive protein, IL-1β and IL-6. The anti-inflammatory cytokine IL-10 was increased in the sildenafil-treated animals, demonstrating a protective effect on the vasodilatory sepsis.
The use of sildenafil appeared to reduce the intensity of the infectious process as total leukocyte and neutrophil counts were lower.

Following these early reports, other groups have investigated PDE5 inhibitors as potential treatment for sepsis. In 2015, a team from the University of Pittsburg, using a mouse CLP model of abdominal sepsis, showed that sildenafil decreased markers of systemic inflammation, protected against organ injury, and increased circulating amounts of tumor necrosis factor receptor 1 (TNFR1) in mice with sepsis [75]. This had the protective effect of limiting inflammation, and the underlying mechanism was reported to be via cyclic guanosine monophosphate (cGMP) signalling. Sildenafil raises levels of cGMP.

The work by Deng et al. effectively linked several strands of evidence for the usefulness of PDE5 inhibitors in treating sepsis. During endotoxemia and sepsis, levels of soluble TNFR1 increase. If TNF-\(\alpha\) is neutralised with sTNFR1, this lessens organ damage [76] and mortality in mice with sepsis [77]. The same laboratory had previously demonstrated that toll-like receptor 4 (TLR4)-dependent expression of the gene encoding inducible nitric oxide synthase (iNOS) in hepatocytes leads to nitric oxide (NO) production and activation of the cGMP- and protein kinase G (PKG)-dependent activation of TNF alpha converting enzyme (TACE), which cleaves TNFR1 [78]. They showed that drugs such as PDE5 inhibitors which increase plasma and cellular cGMP levels are effective in enhancing receptor shedding, reducing systemic inflammation, and protecting organs from injury in sepsis.

In 2017, other groups confirmed the effects of PDE5 inhibitors. Kovalski et al. confirmed the protective effect of sildenafil in the rat CLP model of sepsis featuring significantly increased levels of plasma nitrate/nitrite (NO\(_x\)), aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, creatine kinase and lactate [79]. At a functional level, the sepsis led to hypotension, hyporesponsiveness to vasoconstrictor, and renal blood flow reduction. Sildenafil reversed many of these parameters, and in particular it increased renal blood flow and reduced the plasma levels of creatinine, lactate and creatine kinase, as well as reducing lung myeloperoxidase. Benli et al. reported that another PDE5 inhibitor, tadalafil, has a protective effect on kidney function in a rat model of sepsis [80].

Other organs that can be damaged during sepsis include heart, lungs and brain. What is the evidence for protection of these organs by PDE5 inhibitors?

Sildenafil was originally developed as a cardioprotective agent before being repurposed for male erectile dysfunction. Although definitive evidence was not obtained at the time of its early clinical development, post-launch data has indicated that the drug has cardioprotective properties. The first evidence was provided in 2002 by Ockaili et al. who reported that in the rabbit sildenafil induces protective effects against ischemia-reperfusion injury, which are mediated by opening of mitochondrial K(ATP) channels [81]. Importantly for
cardio-protection in sepsis, the protective effect ‘was powerful within 30 min and persisted to a slightly lesser degree 24 h after administration of the drug.’

Other mechanisms may also come into play in the heart. Fernandes et al. reported that sildenafil protects function of mitochondria in the heart by preventing reactive oxygen species (ROS) generation [82]. Positive effects on mitochondrial function have also been reported by Whitaker et al. who showed that sildenafil treatment can lead to mitochondrial biogenesis following acute kidney injury [83]. Failure of energy production by mitochondria is a major factor in morbidity and mortality from bacterial sepsis so this protective action of sildenafil may be significant.

Kukreja has published a series of studies which expand on the cardioprotective effects of sildenafil including the role of adenosine receptors in its cardioprotective action [84-93].

The only clinical trial reported using sildenafil to treat a severe heart condition in humans is the RELAX trial in Congestive Heart Failure. The drug showed no benefit [94]. A relatively low dose of 20 mg three times a day (TID) was used for the first 12 weeks and this dose was associated with minimal increases in plasma cGMP. The authors concluded that ‘while studies in pulmonary arterial hypertension and HFrEF have observed effects on exercise capacity with similar doses and duration of therapy, we cannot exclude the possibility that inadequate dose or duration of PDE-5 inhibition contributed to our findings.’

There are no reported studies on the effect of PDE5 inhibitors on heart function during bacterial sepsis.

In lungs, the 2011 study by Cadirci et al. in the rat CLP model reported that sildenafil treatment reduced the accumulation of inflammatory cells in lungs leading to less lung injury [73]. Other (non-sepsis) studies of lung injury support this effect. The study by Yildirim et al. investigated the protective effects of sildenafil on tissue integrity, oxidant–antioxidant status, and neutrophil infiltration to the inflamed organ in a rat model of bleomycin-induced lung fibrosis. Treatment of animals with sildenafil (10 mg kg⁻¹ subcutaneously for 14 days) was beneficial with regard to prevention of lipid peroxidation, cytokine (IL-1β and TNF-α) production, neutrophil accumulation, and myeloperoxidase (MPO) activation [95].

In a pig model of sepsis-induced lung injury, Kemper et al. reported that sildenafil administration improved pulmonary hypertension and oxygenation in LPS-induced lung injury but increased shunt fraction and promoted systemic hypotension, an effect that might be deleterious in treatment of sepsis [96]. They concluded ‘it remains unclear whether sildenafil may be beneficial in sepsis patients.’

There have been no reports of animal or human studies on the effect in brain of PDE5 inhibitors in sepsis. Two factors may be relevant: PDE5 in neuronal tissue and PDE5 in microglia. It was recently shown that this enzyme does
occur throughout neuronal tissues (despite earlier doubts) [97] though no studies have been reported on the importance of PDE5 and its inhibitors on neuronal function in sepsis.

In the CNS, microglia have an immune function role similar to that of macrophages in the periphery. Zhao et al. showed that in microglia, sildenafil attenuates LPS-induced pro-inflammatory responses through downregulation of intracellular ROS-related MAPK/NF-κB signaling pathways [98]. Sildenafil markedly inhibited iROS production induced by LPS.

Continuing on the theme of the immune system, recent evidence indicates that sildenafil affects both innate and adaptive immune systems in animals and humans. A thorough first review of the new findings has been provided by Kniotek and Boguska [99]. These authors summarised a growing body of evidence that sildenafil exerts immunomodulatory effects. Sildenafil influences proliferation of regulatory T cells, and production of proinflammatory cytokines and autoantibodies. Sildenafil decreases the levels of proinflammatory cytokines, including TNF-α, IL-1, and reduced NK cell activity, and enhances the action of regulatory T cells. Sildenafil markedly inhibits iROS production induced by LPS. However, the potential immunomodulatory effects of sildenafil in human sepsis remain to be confirmed.

The inflammatory response during sepsis involves myeloid-derived suppressor cells (MDSCs), immature myeloid cells. They are immunosuppressive, suppressing T-cell proliferation and activation. The role of MDSCs in sepsis is hotly debated. It is unclear whether activated MDSCs are beneficial in fighting sepsis through increasing innate immune responses to bacteria, or whether expansion of MDSCs leads to adaptive immune suppression followed by secondary infection. Lai et al. reviewed the role of MDSCs in sepsis [100], including the potential use of sildenafil to inhibit the signal pathways that regulate the production of the suppressive factors of MDSCs. Drawing from research on use of sildenafil to fight cancer cells by boosting the immune system, they noted that this PDE5 inhibitor reduced arginase-1 and nitric oxide synthase-2 expression in a mouse tumor model. It also enhanced intratumoral T-cell infiltration and activation, reduced tumor outgrowth, and improved the antitumor efficacy of adoptive T-cell therapy. Furthermore, sildenafil restored T-cell proliferation of peripheral blood mononuclear cells from multiple myeloma and head and neck cancer patients in vitro [101]. They suggested exploration of potential for similar properties of sildenafil in the context of sepsis.

A potential concern with use of sildenafil in sepsis is that its vasodilator action might lead to exacerbation of arterial hypotension. The drug has only a mild effect on blood pressure, so this may be a minor consideration compared with its protective effects on the microvasculature. Some information from humans is available. In a retrospective analysis of 17 neonates given sildenafil for
treatment of pulmonary hypertension secondary to meconium aspiration syndrome (MAS) or sepsis, no significant decrease in blood pressure or increase in vasopressor/inotrope requirement occurred [102]. However, definitive studies are lacking, and it is currently unknown whether administration of sildenafil to patients with sepsis is efficacious and safe.

9.4.4. Cannabidiol (CBD)

The arachidonic acid derivatives 2-arachidonoyl glycerol and anandamide are endocannabinoids produced excessively in sepsis and they are potential factors leading to immune dysfunction and inflammation [103]. Inflammation is the necessary response of the infected host, controlled by neutrophil recruitment to the site of inflammation [104], however, too much inflammation or unresolved inflammation can lead to many acute and chronic diseases including sepsis [105].

Inflammation and inflammatory disorders, including sepsis, could be modulated by endogenous chemical signalling molecules and their receptors. Among these, endocannabinoids are released in response to inflammatory stimuli and their levels are elevated in patients and animals with septic shock [106]. Three receptors of endocannabinoids have received attention as potential ways to treat sepsis, the CB2 receptor, the transient receptor potential vanilloid subfamily, member 1 (TRPV1), and the GPR55 receptor (The latter has sometimes been referred to as the CB3 receptor). Endocannabinoids suppress immune cell function by binding to G-protein-coupled CB2 receptors on immune cells [107,108]. In vitro, CB2R activation has been shown to inhibit leukocyte proliferation and migration, and promote immune cell apoptosis [109]. Activation of CB2R reduces activation of leukocytes and their interactions with blood vessel walls in septic mice. Also, CB2R activation reduces leukocyte-endothelial interactions and the pro-inflammatory cytokines, TNF, IL-1β and IL-6 in plasma of septic mice [110-112]. CB2R may be an attractive target for modulating the inflammatory response in sepsis.

While the distribution of CB2 receptors is primarily in immune cells, the distribution of GPR55 is extensive throughout the body. Current evidence indicates GPR55 involvement in the gut during septic ileus [113] and in both inflammatory and neuropathic pain [114-117]. In animal models, several groups have investigated the effect of either knock-out of cannabinoid receptors or their modulation by drug-like molecules. Yang used LPS challenge in mice to investigate the therapeutic potential of CB2R and GPR55 modulation [118]. The author concluded that CB2R activation and GPR55 inhibition may
significantly attenuate the hyper-inflammatory response of sepsis and therefore possibly improve patient outcomes, and even reduce mortality.

Others have used different animal models, particularly the cecal ligation and puncture model and also knock-out models in which the CB2 receptor has been deleted. Csoka et al. found that CB2 receptor inactivation by knockout decreased sepsis-induced mortality, and bacterial translocation into the bloodstream of septic animals [119]. Furthermore, CB2 receptor inactivation decreased kidney and muscle injury, suppressed splenic NF-κB activation, and diminished the production of IL-10, IL-6 and MIP-2. CB2 receptor deficiency prevented apoptosis in lymphoid organs and augmented the number of CD11b+ and CD19+ cells during CLP.

However, Tschöp et al. reported the opposite findings [120]. This group also used a cecal ligation and puncture (CLP) model of sepsis, and found that CB2R-knock out mice showed decreased survival as compared with wild-type mice. CB2R-KO mice also had increased serum IL-6 and bacteremia. Twenty-four hours after CLP, the CB2R-deficient mice had increased lung injury, increased neutrophil recruitment, decreased neutrophil activation. CB2R-agonist treatment in wild-type mice increased the mean survival time and decreased neutrophil recruitment, decreased serum IL-6 levels, bacteremia, and damage to the lungs. Gui et al. also proposed that CB2 receptor has a protective action in sepsis [121], and Lehman et al. proposed that CB2 activation reduces intestinal leukocyte recruitment and systemic inflammatory mediator release in acute experimental sepsis [122].

These opposing results leave open the specific contribution of CB2R to sepsis. Kasten et al. provided a possible explanation by suggesting that CB2R mediation of inflammation is dependent upon the severity of sepsis [123]. They suggested that in more severe models of sepsis, the inflammatory response drives hemodynamic changes that result in cardiopulmonary arrest and death. However, in less severe CLP models, inefficient clearance of bacterial pathogens leads to prolongation of the infectious process and resultant death. Thus, “attenuation of inflammation would prove more beneficial in a severe model of CLP, while being harmful in a moderate model of CLP.”

Several papers have reported the effect of the phytocannabinoid drug cannabidiol (CBD) in models of sepsis. CBD is a nonpsychoactive component of marijuana. More than 80 clinical trials have investigated the effects of CBD in various diseases from inflammatory bowel disease to graft versus host disease. It was found to be safe in humans. Chronic administration of CBD for 30 days to healthy volunteers, at daily doses ranging from 10–400 mg, failed to induce any significant alteration in neurological, psychiatric or clinical exams [124,125]. CBD-based formulations are approved for the management of multiple sclerosis in at least 27 countries, and CBD also received U.S. Food and Drug Administration approval for the treatment of refractory childhood
epilepsy and glioblastoma multiforme tumours. CBD appears to act at several receptors including CB2R and GPR55.

In the cecal ligation model in vivo, cannabidiol has shown protective action in several laboratories. In 2010, Cassol et al. provided the first study in the rat CLP model [126]. Rats were treated with CBD (at 2.5, 5, or 10 mg kg\(^{-1}\) once or daily for 9 days after CLP) or vehicle. CBD ameliorated cognitive impairment, and significantly reduced mortality in rats submitted to CLP.

In 2011 Ruiz-Valdepeñas et al. reported using LPS challenge in place of the CLP model [127]. They showed that CBD reduces LPS-induced vascular changes and inflammation in the mouse brain. CBD prevented LPS-induced arteriolar and venular vasodilation as well as leukocyte margination. It abolished LPS-induced increases in TNF-alpha and preserved blood brain barrier integrity.

In 2012, based on a bacterial meningitis model in Wistar rats, Barichello et al. reported that cannabidiol administered by intraperitoneal injection reduced host immune response (TNF-\(\alpha\)) and prevented cognitive impairment [128]. Then in 2014 Ribeiro et al. showed that CBD improves lung function and inflammation in mice submitted to LPS-induced acute lung injury [129]. CBD decreased total lung resistance and elastance, leukocyte migration into the lungs, myeloperoxidase activity in the lung tissue, protein concentration and production of pro-inflammatory cytokines (TNF and IL-6) and chemokines (MCP-1 and MIP-2) in the bronchoalveolar lavage supernatant.

In addition, very recent publications show CBD has beneficial effects on mitochondrial function [130]. It reverses the reduction in mitochondrial capacity induced by poor supply of oxygen and glucose. In the heart, CBD modulates mitochondrial function and biogenesis to protect the heart against doxorubicin-induced cardiomyopathy during cancer treatment [131]. It also protects the heart in studies in mice from auto-immune CD3\(^{+}\) and CD4\(^{+}\) T cell-mediated inflammatory response and injury, myocardial fibrosis and cardiac dysfunction [132]. Similar positive effects on mitochondrial function have been demonstrated in the rat brain [133]. CBD also exerts neuroprotective effects against mitochondrial toxins, and restores intracellular Ca\(^{2+}\) homeostasis in human neuroblastoma cell lines [134]. These data suggest that CBD is a mitochondria-targeting drug that protects and enhances mitochondrial function and bioenergetics, effects that might be relevant in treatment of sepsis.

CBD also has some moderate antibacterial action against Gram-positive bacteria. The bactericidal concentrations of CBD for staphylococci and streptococci in broth are in the range of 1–5 \(\mu\)g ml\(^{-1}\), with activity dropping more than 10-fold in the presence of 4 % serum or 5 % blood. Gram-negative bacteria are resistant to CBD [135].

In summary, endocannabinoids are produced excessively in sepsis and they are potential factors leading to immune dysfunction and inflammation. The
endocannabinoid modulator cannabidiol has shown promising results in laboratory studies and may be a candidate for treatment for sepsis and septic shock. Studies in humans are required to test this possibility.

9.4.5. Immune regulation by (some) antibiotics

Some (not all) antibiotics have additional pharmacological properties in addition to their ability to directly kill bacteria. They can interact with host immune cells and regulate immune function, with potential for beneficial effects in various inflammatory conditions including sepsis. Tetracyclines such as doxycycline and minocycline, and macrolides such as erythromycin, have anti-inflammatory properties due to their ability to regulate the expression of inflammatory cytokines and TLRs in response to LPS [136]. Doxycyclin inhibits LPS-induced NO production by immune cells in vitro [137] and reduces IL-1\(\beta\) and IL-6 expression. Tetracyclines such as doxycycline also possess strong metal chelating properties [138] inhibiting protein kinase C [139]. PKC inhibitors are known to reduce LPS-stimulated cytokine secretion [140,141] therefore the chelating properties of doxycycline might also reduce the IL-1\(\beta\) and IL-6 expression through PKC inhibition. Macrolides such as erythromycin appear to modulate immune function by a different mechanism, by blocking activation of transcription factors NF-\(\kappa\)B and IRF3 [142] with concomitant reduction of both pro-and anti-inflammatory cytokines.

There is extensive evidence for reduction in clinical death rates with macrolide antibiotics beyond that expected from their antibiotic action alone. This has been reviewed by Amsden [143]. For severe lung infections, macrolides are associated with decreased length of hospitalisation and mortality when used alone or in combination with beta-lactam antibiotics [144]. The macrolide azithromycin may modulate inflammation by enhancement of host defence mechanisms shortly after initial administration followed by curtailment of local infection and inflammation in the following period [145].

In the years since publication of the review by Amsden, additional evidence has accumulated for the protective effects of macrolides. A meta-analysis published in 2014 by Sligl et al. found significantly better outcomes with macrolide therapy for critically ill patients with community-acquired pneumonia [146]. In observational studies of almost 10,000 critically ill patients with community-acquired pneumonia, macrolide use was associated with a significant 18\% relative (3\% absolute) reduction in mortality compared with non-macrolide therapies. An even larger mortality reduction was observed after pooling data from studies that provided adjusted risk estimates. The authors concluded that “These results suggest that macrolides be considered first-line combination treatment in critically ill patients with community-acquired pneumonia.”
Similar positive data was reported from a study of use of combinations of antibiotics that included a macrolide in severely ill patients in ICUs. Martin-Loeches et al. conducted a study of intubated patients admitted to the ICU with severe community-acquired pneumonia [147]. This was a prospective, observational, multicenter study conducted in 27 ICUs of 9 European countries. Two hundred eighteen consecutive patients requiring invasive mechanical ventilation for an admission diagnosis of CAP were recruited. Severe sepsis and septic shock were present in 165 (75.7 %) patients. ICU mortality was 37.6 %. Monotherapy was given in 43 (19.7 %) and combination therapy in 175 (80.3 %) patients. Macrolide use was associated with lower ICU mortality (hazard ratio, HR 0.48, confidence intervals, 95 % CI 0.23–0.97, P = 0.04) when compared to the use of fluoroquinolones. When more severe patients presenting severe sepsis and septic shock were analyzed (n = 92), similar results were obtained (HR 0.44, 95 % CI 0.20–0.95, P = 0.03). The authors concluded that “Combination therapy with macrolides should be preferred in intubated patients with severe CAP.”

At least part of the additional effect of macrolides has been shown to be via reduction of inflammatory cytokines [148-151]. Macrolides contain two sugars and their spacing resembles those of the sugars in LPS and also those in the experimental drug PETIM-DG invented by Sunil Shaunak, which blocks interaction of bacterial LPS with TLR4/MD2, giving good protection in a rabbit model of bacterial dysentery [152]. This appears to be a powerful therapeutic mechanism: a study of oral PETIM-DG with no concurrent antibiotic in non-human primates infected with highly lethal Shigella dysenteriae type 1 bacteria gave a high level of protection and reduced death rate [153]. Note that this reduction in death rates was achieved without use of antibiotics. Though PETIM-DG is currently not a marketed drug, this study does highlight the potential for use of alternatives to antibiotics in treatment of serious infections. It remains to be proven whether macrolides modulate the innate immune inflammatory response similarly to PETIM-DG by antagonism of TLR4/MD2.

Macrolide antibiotics have also been shown to inhibit the quorum sensing (QS) ability of bacteria. This action occurs at lower concentrations than their antibacterial activity [154-156]. For example, erythromycin suppresses QS signals of P. aeruginosa including hemagglutinins, protease, hemolysin and AHL signals [157]. Azithromycin also affects QS-regulated virulence genes both in vitro [155-156] and in vivo [158]. It has been suggested than these actions might exploited to enhance antibiotic therapy by targeting bacterial behaviour [159].

The use of antibiotic combinations for joint effects on QS and cell kill remains a relatively little explored opportunity.

Tetracycline antibiotics also have non-antibiotic properties that might be exploited in severely ill patients. Evidence is available showing protective effects in the brain, particularly by modulation of the inflammatory actions of
microglia, which fulfil an innate immune role in brain similar to that of macrophages in the periphery. Microglia mediate inflammation in various infectious and neurodegenerative diseases. The ability of minocycline to inhibit microglia has been studied for example in LPS damage to retina [160], global cerebral ischemia [161], focal cerebral ischemia [162-164], and traumatic brain injury [165,166]. Studies of the effect of minocycline in animal models of sepsis have shown that it decreases brain inflammation induced by systemic and brain administration of LPS [167,168]. In a rat CLP sepsis model, minocycline prevented an increase in markers of oxidative damage and inflammation in the hippocampus after sepsis. This was associated with improved long-term cognitive performance in sepsis survivors [169]. Blockade of microglia activation by minocycline was able to decrease not only hippocampus cytokine levels but also the disruption of the BBB, perhaps because minocycline decreases the activation of brain metalloproteinases. MMP activation is a pivotal step in disruption of the BBB [170,171].

In summary, macrolide and tetracycline antibiotics have significant additional properties beyond killing bacteria. Their effects in vivo on inflammation, quorum sensing and protection of organs including the blood brain barrier suggests opportunity for ‘repurposing’ in combinations with other classes of antibiotic to extend opportunities for overcoming antibiotic resistance.

9.5. CALL TO ACTION

This paper has summarised several opportunities to exploit drugs that are currently available to help reduce death rates from antibiotic resistance. These opportunities do not depend on the difficult, slow and costly task of inventing new antibiotics, and they could be tested and implemented very rapidly to fill the (probably large) gap in time before new chemical and mechanistic classes of antibiotics become available. The overall theme is that repurposed drugs used in combinations could be explored more rigorously to reduce resistance and preserve antibiotic efficacy, and to protect organs from damage leading to disability and death. The key facts and actions required are summarised here:

i) The idea of using combinations of (non-antibiotic) beta-lactamase inhibitors as resistance-breakers appears to be unexploited. Several combinations of beta-lactam antibiotic plus lactamase inhibitor are commercially available and these number among our most valuable antibiotics, yet there are no available combinations of a beta-lactam antibiotic with two or more non-antibiotic lactamase inhibitors. Also, the possibility of combining two antibiotics with a lactamase inhibitor appears to be totally unexplored. If proven effective, these ideas alone could transform treatment options against our most serious bacterial infections.
ii) The fact that only a single combination of two antibiotics has been approved for treating Gram-negative bacteria is shocking. (Co-trimoxazole is the only combination registered by the FDA and EMA that treats Gram-negative bacteria). Combinations of drugs have been thoroughly explored and registered for use to improve efficacy and overcome resistance for infectious diseases such as tuberculosis, HIV, and hepatitis C, but not against systemic infections by Gram-negative and Gram-positive bacteria. There are large numbers of available antibiotics that could be explored in combinations of two or three for synergistic effects and ability to overcome bacterial resistance.

iii) The additional non-antibiotic properties of macrolides and tetracyclines offer opportunity to use these drugs in combinations to reduce death rates in clinical practice. These properties should be kept in mind when selecting combinations of antibiotics as suggested above. Simple bacterial kill assays will not help detect these effects. Relevant assays both in vitro and in vivo need to be used more routinely in the antibacterial field. Over-reliance on simple in vitro bacterial kill assays may be limiting the discovery of break-through treatments. If these antibiotics could be shown useful in vivo when used in combinations with other antibiotics against some of the most serious resistant bacteria, it could allow lower use of beta-lactam antibiotics and possibly side-step the concern about CREs.

iv) The lethality of bacteria derives from the damage to organs of the body. Strategies to protect organs during infection remain little explored. Particularly, reduction in deaths from sepsis and septic shock may depend on non-antibiotic interventions designed to protect kidneys, heart, lung, brain and other organs. Several options have been summarised in this review. Additional scientific publications not covered here suggest that other organ-protecting drugs and nutraceuticals might be available for repurposing; these require additional investigation. Use of these repurposed drugs alongside antibiotics could reduce death rates. Recovery from sepsis could be faster, allowing reduction in use of antibiotics. This could help slow development of antibiotic resistance globally and make our precious antibiotics last longer.

v) Moreover, current guidelines for treatment of sepsis and other indications do not account for the impact of antibiotics (positive and negative) on the peripheral and central immune systems, despite widespread immune dysregulation in sepsis. The potential positive effects of macrolides and tetracyclines have been summarised above. This article has highlighted the positive effects of some antibiotics on the immune system, but it is also possible that some antibiotics have unrecognised negative effects on for instance immune-regulated inflammation, quorum sensing, organ protection or gut microbiome.
which could work against curing the patient. Further study of these immune modulatory effects of antibiotics could lead to change in treatment of sepsis and other conditions. Again, this emphasises that simple bacterial-kill assays need supplementing with more-sophisticated in vitro and in vivo assays that give a realistic view of the impact in humans of antibiotics and other treatments.

vi) Strategies to side-step the emerging crisis of CRE’s and other bacteria with ESBLs and MBLs should be explored by use of combinations of antibiotics excluding beta-lactams. If host-mediated mechanisms can be exploited to boost host defence and protect organs, this could include replacement of bactericidal antibiotics such as beta-lactams, aminoglycosides, and fluoroquinolones with bacteriostatic antibiotics such as macrolides, tetracyclines and sulphonamides. A meta-analysis showed no reduction in cure rates against abdominal, lung and soft tissue infections when bacteriostatics were compared with bactericidals [16].

Antibiotics in use today were discovered using 20th century technologies. The past decade has delivered many significant advances in technology that could help with maximising benefit from currently available drugs, such as the internet, smart-phones, Electronic Health Records, genomics/ metabolomics/ transcriptomics, big data and machine learning. Initiatives such as CMAP at the Broad Institute, which established the first collection of genome-wide transcriptional expression data from drug-treated human cells, are beginning to guide drug repurposing efforts [172]. Internet-based connectivity allows discovery of unexpected connections in health data, leading to rapid implementation of new treatment regimes, as beautifully illustrated in a TEDmed talk [173]. Also, the internet is now offering global research platforms to foster collaborative research, illustrated by Worldlabs and the global S.E.P.S.I.S. community [174].

In closing, there is real reason for concern about antibiotic resistance, but invention of new antibiotics is not the only solution. Repurposing opportunities for antibiotics and non-antibiotics described in this article (together with additional ideas summarised in an earlier review by this author [175]) may be able to fill the gap before new antibiotics classes are discovered.

**AUTHOR DECLARATION OF INTERESTS**

I was named co-inventor on the patent for sildenafil / Viagra. I have no commercial or monetary interest in the drug. I have no monetary interest in any of the other drugs mentioned in this article.
The only way to fill the gap: combinations of repurposed drugs against ...
63. [https://emcrit.org/pulmcrit/metabolic-sepsis-resuscitation/](https://emcrit.org/pulmcrit/metabolic-sepsis-resuscitation/)
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Chapter 10

ANTIMICROBIAL COMPOUNDS FROM PLANTS

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10.1. INTRODUCTION

The increased spread of drug-resistant microorganisms, due to the indiscriminate and irrational use of antibiotics, has become a current threat in the therapy of microbial diseases that these days has led to the selection of new resistant strains of bacteria, genetically changed. It is known that antibiotic resistance is generally caused by spontaneous mutations in specific genes, but bacterial drug resistance mechanisms are more complex than they appear. In many cases, the effectiveness of common antibiotics is lost after a period of a few years. The World Health Organisation (WHO) reports that there are internationally high levels of antimicrobial resistance (AMR) in common bacteria alongside limited understanding and uncoordinated surveillance of AMR [1]. In the last decades there is a more prevalent resistance in cases of bacterial infections such as respiratory tract infections, diarrhea, meningitis, syphilis, gonorrhea and tuberculosis [2]. Staphylococcus aureus isolated from clinical samples are now showing resistance to more than three drugs and are considered as multiple-drug resistant bacteria [2] Antibiotic resistance is a natural phenomenon, but the abuse and overdose have caused numerous resistance problems. The rise of multidrug resistance (MDR) pathogens seems to be caused by the continuous selective pressure, and the emergence of new survival strategies of bacteria are in response to the new classes of antibiotics [3]. In addition, the incorrect and inappropriate use of antibiotics has increased the prevalence of resistant bacteria. Also, for fungi and protozoa, current chemotherapeutic options are very limited and characterized by side effects or toxicity.

If the situation remains unchanged, the number of deaths attributable to MDR will be more than 10 million in 2050, higher than the number of cancer-associated deaths (8.2 million per annum) [3]. On average, the cost of producing a new antibiotic is higher than the cost in the 1990s by up to 60% [3].

Consequently, researchers are currently looking for new sources of antibiotics with a broad spectrum of action against Gram-negative and Gram-positive bacterial strains, with as few as possible side effects [2]. The search for new therapeutics related to the urgent development of new, more effective drugs and to the eradication of MDR pathogens that can cause deadly infections represents a major problem for present and future.

A renewable source of antibacterial, antifungal and antiparasitic compounds is represented by plants, belonging to the most different genera. Plants are characterized by a large structural and functional diversity of their compounds synthesized in all morphological parts as secondary metabolites, with various functions in plant survival.
Many of the contemporary pharmaceutics, cosmetics, and food industries are founded on the knowledge of the properties of medicinal plants. The antimicrobial compounds extracted from plants can be used for applications in treating infectious, systematic and inflammatory diseases, in food preservation and agriculture, against phytopathogens [4].

New plant compounds with diverse chemical structure and mechanisms of action have been extensively studied by ethnopharmacologists, botanists, microbiologists and chemists. These phytochemicals are considered to be effective, safe, and natural compounds, with no or lower side effects. During the growing stages, plants synthesize a wide variety of secondary metabolites, such as flavonoids, alkaloids, tannins, terpenoids, peptides and others, which have been found in vitro to have antimicrobial properties. The antimicrobial activity of plant-derived extracts is correlated with the plant species, local climatic and environmental conditions, harvesting conditions, and extraction technology [5].

In food industry, the antimicrobial properties of many aromatic plants were used for the control of spoilage and harmful pathogenic bacteria, along with chemical preservatives, whose use is limited due to their toxicity, carcinogenity, teratogenicity and environmental problems. The public opinion about chemical antimicrobial additives has become unfavorable and generated interest in the use of natural compounds [6].

10.2. HISTORY

The importance of medicinal plants was acknowledged at least 50,000 years ago, as evidenced by numerous archaeological excavations. The oldest medical document — the Ebers papyrus — dated to the 15th century BC, contains 800 recipes using herbs and different extracts. In a copy of the book Pen-tsao, a document describing medicinal plants used in China and the Far East, dated 7th century AD, nearly 400 medicinal herbs used in juices, infusions and ointments are described [7].

In India, the traditional Ayurvedic concept mentioned in the ancient Vedas was developed between 2500 and 500 BC and represents the ‘science of life’ and the ‘science of longevity’. The Ayurvedic system promotes the use of medicinal herbs and extracts, and other special diets, exercise and lifestyle recommendations.

In the culture of Chinese, Indians, Egyptians, Romans, and Arabs, herbalism was integrated as a philosophical principle. In ancient Europe, herbal medicine was discovered later, in Greece, and was mentioned by Hippocrates of Kos. The document Corpus Hippocraticum presents the beneficial effects of more than 400 species of plants. Another Greek philosopher, Theophrastus of Eresos,
Antimicrobial compounds from plants

considered to be the father of botany, studied more than 500 plants. In 40–90 AD, Dioscorides wrote *De Materia Medica* containing a series of herbal treatments. In ancient Rome, Claudius Galenus (130–200 AD) studied 450 plants and the formulations of plant medication. After the fall of the Roman Empire, the Arabs introduced new medicinal plants and flavoring agents such as cloves, vanilla, camphor and nutmeg. Avicenna, the physician and philosopher, wrote nearly 500 books containing more than 700 herbal treatments. Paracelsus (1493–1541) claimed that specific substances extracted from plants are involved in the treatment of disease [7].

In the Middle Ages the cultivation of medicinal plants in abbeys and monasteries facilitated the study of therapeutic properties. In the period of the Ottoman Empire hospitals were built near Orthodox monasteries, where the medicinal plants were used in the treatment of disease.

The methods used for extraction allowed the preparation of many different drugs, and, to this day, a lot of research has been done concerning the separation of different compounds from plants. At the same time, the synthesis of plant substances was studied. The obtaining of salicylic acid from willow is considered now as the beginning of pharmaceutical industry.

With the development of medicine, plant extracts have begun to be replaced with synthetic drugs, considered more effective and easier to produce. Nowadays, the use of medicinal plants has been re-examined and the active principles and action mechanisms have been researched in numerous studies. The current tendency to use, in addition to chemical substances of synthesis, herbal preparations is increasing, and this is explained by their antimicrobial, antioxidant, anticancer and other functional properties [4].

### 10.3. CLASSIFICATION OF ANTIMICROBIAL COMPOUNDS

In addition to the primary metabolites (proteins, carbohydrates, fats), that play a major role in maintenance of plant viability, a series of compounds including terpenes, polyphenols, quinones, alkaloids, and peptides, which belong to the secondary metabolism, are also synthesized. In fact, the separation between primary and secondary metabolism is uncertain, as many of the primary metabolic intermediates perform similar roles in secondary metabolism. Secondary metabolites are present only in certain species, often exhibiting organ or tissue specificity, and can only be identified at a certain stage of growth and development within a species or may be activated only during periods of stress caused for example by the attack of microorganisms or nutrient depletion.

Their synthesis (Figure 1) seems to have no direct meaning for the plant cell, but it can be decisive for the development and functioning of the plant organism as a whole. As a result, given their conservation during the evolution...
of the plant kingdom, it is highly plausible to suggest that secondary metabolites offer a selective advantage to species.

Plants remain the only sources of extraction of some compounds, since many of the secondary metabolites cannot be synthesized chemically, being complex stereostructures with many chiral centers that may be essential for biological activity. Out of the secondary metabolites, up to 12,000 were isolated, representing less than 10 % [8].

![Figure 1. General scheme of biosynthetic pathways and precursors for the major classes of secondary metabolites [9]](image-url)

Many plant secondary metabolites with a defensive role are secreted externally by epidermal cells or epidermal formations such as glandular hairs, that may be short or long, single or pluricellular. Many multicellular hairs (trichomes) secrete a variety of compounds, such as volatile oils, alkaloids, oleoresins, resins, and balms.
In *Mentha piperita* (*Lamiaceae* family), glandular hairs are usually formed from several secretory cells, on a short pedicel inserted into an epidermal intrusion (Figure 2).

![Figure 2. Glandular hairs in a) Mentha piperita and b) Artemisia dracunculus](image)

In plants, the functional metabolites are grouped into different classes based on chemical structure and similar properties.

### 10.3.1. Polyphenols

Polyphenols are one of the most important and at the same time the most numerous of the secondary metabolite groups, omnipresent in the plant kingdom. At present, over 8,000 phenolic structures have been identified in a wide variety of forms, of which more than 4,000 belong to the flavonoid class, and of these, several hundred are present in edible plants [8]. Of polyphenols, a series of pigments with the quinonic structure are responsible for the color of fruits and flowers (alizarin, purpurin, benzoquinone, juglone).

Phenolic compounds are very important for plants and can have multiple functions. These molecules are generally involved in the defense against ultraviolet radiation, oxidizing agents or the aggression of some phytopathogenic agents [8], and they have a role in adapting to biotic and abiotic stress.

Polyphenols can be classified into different groups according to the number of phenolic rings they contain and the structural elements linking these rings. They are classified in phenolic acids, flavonoids (flavonones, flavones, xanthones and catechins, anthocyanins, anthocyanidins), lignans, stilbens and other polyphenols with non-flavonoid structure.
Phenolic acids are phenols possessing a carboxylic functional group, varying due to the hydroxylation or methoxylation of aromatic nucleous. Gallic acid, which is also part of the composition of hydrolysable tannins, and vanillic acid are present in almost all plants. Caffeic acid (Figure 3) is considered to be the most common phenolic compound distributed in the plant kingdom, followed by chlorogenic acid, which is known to cause allergic dermatitis. Phenols are essentially a series of natural antioxidants, used as nutraceuticals. However, it is assumed that the total content of polyphenols in plants is underestimated and many of the phenolic compounds and their derivatives have not yet been identified due to limitations of the methods used and techniques of analysis [2,3,7,10]. Phenolic compounds possessing a C3 side chain at a lower level of oxidation and oxygen-free levels are classified as essential oils and also have antimicrobial action. Eugenol in clove oil is well known as having an inhibiting action against bacteria and fungi.

Catechol and pyrogallol are hydroxylated phenols, toxic to microorganisms. Catechol (Figure 3) has two OH groups and pyrogallol has three. The mechanisms of action responsible for phenol toxicity against microorganisms refer mainly to the inhibition of enzymes by oxidative compounds, possibly by reaction with sulphydryl groups or by several other non-protein interactions [8]. In 2006 Kocacaliskan et al. investigated the antimicrobial activity of catechol and pyrogallol against three bacterial species, namely *Pseudomonas putida*, *P. pyocyanea*, and *Corynebacterium xerosis* and two fungal species, *Fusarium oxysporum* and *Penicillium italicum*, phytopathogenic species. Using the disc diffusion method, the authors demonstrated that bacteria were inhibited at 5 mM concentration of catechol and pyrogallol, but only catechol had an effect against the tested fungi [11]. Methanolic extract of *Diospyros kaki* Thunb. roots, containing catechol, inhibits the growth of *Clostridium difficile*, *C. perfringes* (significant inhibition for a dose of 5.0 mg/disc), *Escherichia coli* (moderate inhibition), but did not inhibit *Bifidobacterium breve*, *B. longum* and *Lactobacillus casei*. The authors claimed that *D. kaki* root-isolated catechol and its derivatives (4-nitrocatechol, 4-tert-butylcatechol, tetrabromocatechol) could be useful as preventive agents against diseases caused by harmful intestinal bacteria [12].

### 10.3.2. Resveratrol

A very studied compound in the last decades is resveratrol, especially known for its antioxidant properties. Resveratrol (3,5,4′-trihydroxystilbene) is a small polyphenol with 228 g mol\(^{-1}\) molecular weight, in the same time a stilbenoid compound (hydroxylated stilbene derivatives) that was first isolated in the root of white hellebore, *Veratrum grandiflorum* by M. Takaoka in 1939 [22]. Resveratrol production is stimulated by pathogens, ultraviolet (UV)-irradiation, and exposure to ozone, being considered a phytoalexin [44]. This
compound has shown antibacterial, antiviral and antifungal activity and seems to possess a significant role in regulation of immune systems, chemoprevention, neuroprotection, cardioprotection, diabetes prevention and lipid regulation. This antimicrobial compound is produced in more than 70 particular families of plants including peanuts, grapevines, pines, different kinds of berries, legumes and grasses, Scots pine (*Pinus sylvestris*), and other plants [13].

Resveratrol has antiviral activity against hepatitis C virus, respiratory syncytial virus, herpes simplex virus, varicella zoster virus, influenza virus, human immunodeficiency virus etc. The antiviral effect is explained by the inhibition of viral replication, nucleic acid synthesis, protein synthesis, and gene expression. The antifungal effect of resveratrol is demonstrated against pathogenic fungi like *Pyricularia oryzae*, *Plasmopara viticola*, *Cladosporium cucumerinum*, and *Sphaeropsis sapinea* [13,14], for which the mechanism of action is unknown. In *Candida albicans*, resveratrol could penetrate the membrane and induce apoptosis through activated metacaspase and promoted cytochrome c release [15]. The antibacterial effects of resveratrol have been demonstrated in *E. coli* O157:H7 [16], *Salmonella typhi murium* [17], *Listeria monocytogenes* [18], *S. aureus*, *Vibrio cholerae* [17], *Pseudomonas aeruginosa* [19], *Campylobacter jejuni*, *Mycobacterium tuberculosis* [20], methicillin-resistant *S. aureus* (MRSA) [21], vancomycin-resistant Enterococcus (VRE) [20], *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and others. Resveratrol has bacteriostatic activity rather than bactericidal activity [22].

![Chemical structures of representatives of phenolic derivatives](image)

**Figure 3.** Chemical structures of representatives of phenolic derivatives

### 10.3.3. Flavones, flavonoids, and flavonols

Flavonoids are phenolic compounds found in land plants like bryophytes (hornworts, liverworts, mosses) and vascular plants (ferns, gymnosperms, angiosperms). They are synthesized in the cytoplasm of the plant cell and then accumulate in vacuoles that fuse with the central vacuole of epidermis and cortex cells. Flavonoids have a defensive function against insects, fungi and viruses, as well as against invading invertebrates.
The biological action of flavonoids is due to their ability to complex soluble and extracellular proteins and to bind to the bacterial cell wall. It has been shown that several lipophilic flavonoids may disrupt cell membranes [23,24].

Flavones (Figure 4) are flavonoids having an unsaturated 3-C chain with a C2-C3 double bond, such as flavonols, which differ in the absence of a hydroxyl group at position 3. This minor difference in structure has major consequences in biosynthesis and the physiological role of the flavones in the cell. Flavones are found especially in vascular plants in which they form glycosides and aglycones.

![Chemical structures of some representative flavones and flavonoids](image)

**Figure 4.** Chemical structures of some representative flavones and flavonoids

### 10.3.4. Catechins

Catechins are the most reduced form of the C3 unit in flavonoid compounds and have been extensively researched due to their presence in oolong tea and other types of tea. These compounds have been shown to have antimicrobial action against *Streptococcus mutans* [25], *Shigella* [26], *Vibrio* [27] and other pathogenic bacteria [8].

### 10.3.5. Quinones

Quinones are composed of aromatic rings with two ketone substitutions, divided into four classes, namely benzoquinones, naphtoquinones, phenanthrenequinones, and anthraquinones, according to the number of benzene rings in their structure (Figure 5).

Quinones are widespread in plants and mainly exist in higher plants such as *Polygonaceae*, *Rubiaceae*, *Leguminosae*, *Rhamnaceae*, *Labiatae*, and *Boraginaceae* families. A large number of quinones are synthesized in plants via the shikimate or polyketide pathways [8].

A large number of quinones possess significant biological activities, such as the antibacterial and anticancer activities of juglone and plumbagin isolated from *Juglans* and *Plumbago* [28] species. Juglone showed antimicrobial effect against
S. aureus by reducing cell wall formation and increasing membrane permeability [29].

S. Inouye demonstrated the antimicrobial activity of five terpenoid quinones from Monarda fistulosa against Trichophyton mentagrophytes, one of the major dermatophytes causing tinea infections in humans [30].

Quinones are very reactive due to their structure. They are colorful compounds responsible for browning reactions in fruits and vegetables. They are intermediaries in the synthesis of melanin in the skin [8]. In addition to antimicrobial activity, these compounds exhibit numerous other biological activities such as neurological, antiplasmodial, antioxidant, trypanocidal, antitumor, and anti-HIV. In particular, anthraquinones have a broad spectrum of antibacterial activities (including antimycobacterial) based on the inactivation and loss of bacterial protein functions, such as adhesins, cell wall polypeptides, and membrane enzymes [31]. Habbal et al., in 2011, reported the presence of quinine in Lawsonia inermis used for henna extract, with antimicrobial activity against P. aeruginosa [32]. Hypericin, an anthraquinone from Hypericum perforatum, possess antimicrobial properties against methicillin-resistant and methicillin-sensitive Staphylococcus [46].

The increased activity and reactivity of the quinones are due to the redox properties of the carbonyl groups. The reversible transformation between diphenol (or hydroquinone) and diketone (or quinone) easily results in redox reactions. The electrophilic nature of quinones is one of the causes of quinone toxicity for microbial cells. In addition, in biological systems, quinones can cause toxicity through the formation of reactive oxygen species (ROS). Superoxide anion radicals (O$_2^-$) can trigger spontaneous reactions or enzymatic reactions and generate hydroxyl radicals that alter the structure of proteins, by oxidizing nucleophilic amino acids such as cysteine. Also, these oxidation reactions can affect membrane lipids by forming lipid hydroperoxides. They give characteristic precipitation reactions with numerous reagents (potassium iodide, platinum chloride, picric acid, sulfuric acid, etc.).

![Figure 5. Chemical structures of representative quinones, quinone and hypericin](image-url)
10.3.6. Alkaloids

Alkaloids exert considerable physiological effects on humans and animals and are used in therapeutics as narcotics and have a calming effect. Some alkaloids are strong poisons, for example, curare, which has the ability to paralyze the nervous system. Alkaloids are considered to be an insecticide defense means in plants. Some alkaloids, for example nicotine, play a role in enzymatic oxidation-reduction processes.

The alkaloid-producing plants are dicotyledons, and to a lesser extent monocotyledons and cryptogamas. Generally, a plant contains several alkaloids. The alkaloid content depends on the age of the plant, region, climate and season. Algae and bryophytes do not produce alkaloids. They are rare in mushrooms (ergotamine), in pteridophytes (nicotine in *Equisetum*, conine in *Lycopodium* spores) and in gymnosperms (ephedrine). In the angiosperms, some families are reputed by their high contents in alkaloids: *Solanaceae* (solanine, nicotine, hyoscyamine, atropine), *Rubiaceae* (caffeine, quinine), and *Papaveraceae* (morphine, papaverine, codeine, narceine, etc.). The most important alkaloids are: coniine, nicotine, tropane, atropine, cocaine, quinine, papaverine, morphine, codeine, strychnine, caffeine, and others [3,8].

Alkaloids have a great structural diversity and are synthesized mainly in *Solanaceae* and *Fabaceae*. For example, pepper (*Capsicum annuum*), contains alkamide alkaloids such as affinin (spilanthol) and capsaicin [33], potato (*Solanum tuberosum*) contains several toxic and teratogenic glycoalkaloids [34], and lupin (*Lupinus angustifolius*) contains a large number of quinolizidine alkaloids, notably 13 α-hydroxy lupanine and lupanine. Highly unsaturated planar quaternary alkaloids possess the ability to intercalate with DNA [8] and modify the nucleotide sequences [10].

*Tamarindus indica* (Fabaceae) aqueous pulp extract was found to have antimicrobial effect against *E. coli*, *S. aureus*, and *P. aeruginosa*, but not against *Salmonella typhi* [35]. *Zapoteca portoricensis* leaf extract has antibacterial action against Gram-positive bacteria *S. aureus*, *E. coli*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*, and Gram-negative *P. aeruginosa* [35].

10.3.7. Lignans

Lignans are a group of dimeric phenylpropanoids discovered in 1948 by Howarth. Lignans are widespreaded throughout plants in pterydophytes, gymnosperms and angiosperms and are considered one of the earliest forms of defense in vascular plants. Lignans are found in six families of the Coniferae order of Gymnosperms [36]. Cupressaceae and Pinaceae contain the largest variety of compounds. Lignans are also found in Taxaceae, Ephedraceae and Ginkgoaceae [37].
Styraxjaponoside C isolated from *Styrax japonica* (Figure 6) presented an antifungal effect against the human pathogenic yeast *C. albicans*, with membrane-active mechanisms [38]. Lignans isolated from *Pseudolarix kaempferi* showed antimicrobial activity against *C. albicans* and *S. aureus* [39]. Dibenzocyclooctadiene lignin isolated from *Schissandra chinensis* was reported to inhibit *Chlamydia trachomatis* and *C. pneumoniae* [40].

![Chemical structure of styraxjaponoside](image)

**Figure 6.** Chemical structure of styraxjaponoside

### 10.3.8. Glycosides

Glycosides are organic compounds in which a sugar is bound to another functional group through a glycosidic bond (Figure 7). According to the type of aglycone, glycosides are classified in phenolic glycosides and cyanogenic glycosides (aglycone represented by a cyanide group). In many plants chemicals are in the form of inactive glycosides, which can be activated by enzyme hydrolysis. An important group of glycosides are the glucosinolates, the precursors of isothiocyanates, found in 16 dicotyledonous families [10].

Glucosinolates are secondary metabolites that contain sulphur and nitrogen, mainly found in the *Brassicaceae* family. Sinigrin is present in broccoli, mustard and Brussels sprouts and is reported to have antifungal, antimicrobial, anticancer, antioxidant and anti-inflammatory activity. *Lobularia libyca* produces glucosinolates such as glucoiberverine, glucoiberin and glucoerucin with antimicrobial activity against *C. albicans* and *P. aeruginosa* [2].
10.3.9. Saponins

Saponins are compounds derived from steroids or triterpenoid glycosides, which occur in many plants and act on microbial cells by permeabilization of the membrane. Saponins are found in *Solanum* [41], shoots of oats (*Avena sativa*) [42], seeds of *C. annuum* [43], and *Medicago* sp. [44]. These saponins have been tested against several Gram-positive and Gram-negative bacteria, yeast, and fungi, and many of them exhibited a weak growth inhibition against the microorganisms. Saponins from *Yucca* (Figure 8) exhibit antimicrobial activity against Gram-positive bacteria but do not affect Gram-negative bacteria [10].

10.3.10. Tannins

Tannins are polymeric phenolic compounds, water-soluble, astringent, with molecular weight ranging from 500–3000, with different biological activities.
Vegetable tannins are substances well known for their many practical applications, especially in traditional medicine and the tanning industry. The tanning operation of animal skin with plant tannins has been known since antiquity and was done using the bark of different trees, especially oak species [8].

The determination of tannins’ molecular structure allowed differentiation in the practical use between different types of tannins. Tannins (Figure 9) are classified as 1) hydrolysable tannins and 2) condensed tannins. Hydrolysable tannins are based on gallic acid and contain esters of gallic acid with D-glucose. By acid hydrolysis or in the presence of tannases, these tannins release a sugar, usually glucose, and gallic acid (gallotannins) or a compound related to gallic acid such as m-digallic acid or ellagic acid (ellagotannins).

Condensed tannins (proanthocyanidins) are substances derived from flavonoids. The biosynthesis of these metabolites occurs either by condensation of flavan derivatives or by polymerization of quinones units. They seem to play a role in plant protection against insects and ruminant animals [4,8].

Tannins are synthesized in all organs of the plant, especially in leaves, roots and stems. They are found in large quantities in bark, wood and oak leaves, alder, spruce, poplar, walnut, blueberries, mangroves and in the fruits of some species like Terminalia chebula, Caesalpinia brevifolia, etc. [45]. In plants, tannins play an important biochemical role. They increase the resistance of plants to viruses and microorganisms. Consumption of tannins from plant products may have an impact on physiological activities: tannins can stimulate phagocytic cell activity, have host-mediated antitumor activity, have antibacterial and antifungal activity in some infectious diseases.

Their mechanism of action can be explained by forming complex proteins through physical bonds and hydrophobic effects, but they can also form covalent bonds with proteins. In this way, tannins can inactivate functional cell proteins such as adhesins, enzymes, and transport proteins in the cell envelope [8,34].
10.3.11. Terpenes and essential oils

A very interesting and studied group of antimicrobial compounds in plants are essential oils. Essential oils are natural volatile liquids found in flowers, roots, barks, leaves, seeds, fruits, and wood [46]. The International Organization for Standardization (ISO) has defined essential oils as a ‘product obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase if any by physical processes’ [47].

Essential oils (Figure 10) are aromatic compounds insoluble in water but soluble in organic solvents. The composition of essential oils varies depending on plant species and other climatic factors and has a significant role in plant defense and pollination. Essential oils are extracted by hydrodistillation, extraction in organic solvents or in supercritical fluids such as supercritical CO₂ [25].

The antimicrobial activity of essential oils can be explained by their composition: about 90-95% are monoterpenes and sesquiterpene hydrocarbons and their oxygenated derivatives, aldehydes, alcohols and esters. In the rest of the non-volatile part there are hydrocarbons, fatty acids, sterols, carotenoids, waxes, cumarines and flavonoids. In this mixture of compounds in essential oils, the most active against bacteria and fungi are: terpenes (e.g., p-cymene, limonene), terpenoids (e.g., thymol, carvacrol), phenylpropenes (e.g., eugenol, vanillin) and other compounds such as allicin or isothiocyanates [48].
Antimicrobial compounds from plants

Figure 10. Chemical structure of a) linalool; b) limonene; c) α-pinene; d) artemisin

The main mechanisms of action for essential oils are presented in Figure 11 [46]. Essential oils interact with the lipids in a cell membrane and can pass easily in the cytoplasm, due to their lipophilic character. The simpler structure of cell wall in Gram-positive bacteria allows the interaction with the compounds of essential oils, while the more complex structure of cell envelope in Gram-negative bacteria is like a barrier through which the essential oils pass with more difficulty [49].

Figure 11. Mechanisms of antimicrobial activity of essential oils [46]
The antimicrobial activity of essential oils is based on the hydrophobicity, disturbance of the cytoplasmic membrane, disruption of the electron flow, active transport, and coagulation of cell contents. Other mechanisms include disturbances of the pH gradient and the electric potential of the proton-motive force [46]. The great effect of essential oils is due to the lipophilic nature of the hydrocarbon skeleton and hydrophilic nature of functional groups. The most active molecules are the phenolic compounds, following in order by aldehydes, ketones, alcohols, ethers and hydrocarbons [50], that interfere with the cell membrane and with the enzymes involved in energy production. The shape of the bacteria may influence the effect of essential oils, and it has been demonstrated that the rod-shaped cells are more susceptible than coccus-shape [4,49].

The volatile oils of black pepper (Piper nigrum L.), clove (Syzygium aromaticum (L.), geranium (Pelargonium graveolens L’Herit), nutmeg (Myristica fragrans Houtt.), oregano (Origanum vulgare ssp. hirtum (Link) Letsw.) and thyme (Thymus vulgaris L.) were screened for antimicrobial activity against 25 microorganisms of significant importance. The volatile oils exhibited considerable inhibitory effects against all the microorganisms, the oil with the widest spectrum of activity being the extract of T. vulgaris [51].

Sakkas et al., in 2017 [4], studied the correlation between the composition and the antimicrobial efficacy for basil, oregano, and thyme oil and demonstrated that their activity is attributed to different compounds. In basil antibacterial activity of essential oil is due to its high content in linalool and estragole, whereas the antimicrobial spectrum is restricted to specific bacteria (Staphylococcus spp., Enterococcus spp., E. coli, P. aeruginosa, A. baumannii, A. hydrophila, B. cereus, B. subtilis, Enterobacter spp., Listeria spp., Proteus spp., Salmonella spp., Serratia marcescens, and Y. enterocolitica) and fungi (Candida spp., Rhodotorula spp., and S. cerevisiae). The antimicrobial effect of oregano oil is accredited to carvacrol and thymol, and its antimicrobial spectrum is broad, including among others several species of harmful bacteria (methicillin-resistant S. aureus, Listeria innocua, L. monocytogenes, A. baumannii, K. pneumoniae). The antimicrobial effect of thyme oil is also attributed to carvacrol and thymol.

The effect of essential oil from Mentha piperita on the hyphal cell wall and sporulation in fungi is visible on the optical microscope (Figure 12).
10.3.12. Peptides

In their defense against pathogens, plants synthesize both proteins with an enzymatic role (glucanases, proteinases, amylases, oxydases) as primary metabolites, but also peptides with a lower molecular weight of about 10 kDa, known as antimicrobial peptides (AMPs). AMPs have been isolated from a large number of plants and were classified into several categories according to the secondary structure and the three-dimensional conformation (Figure 13). These groups are: 1) linear $\alpha$-helical peptides; 2) cyclic peptides with $\beta$-sheet structures and disulfide bonds; 3) $\alpha$-helix combined with $\beta$-sheet, linked through disulfide bonds; 4) peptides with $\beta$-hairpin or looped arrangement with disulfide bonds; 5) linear peptides containing predominant amino acid residues as proline, glycine, tryptophan and histidine; and 6) small peptides with coil or undefined side structures [53].

A large number of AMPs are characterized by a large number of specific amino acids, such as cysteine, which allow the stabilization of peptide structures by disulfide bridges ($\text{S-S}$). Such examples are defensins and snakins [54]. Small-sized molecules, positive charge and high hydrophobic zones give AMPs specific properties and a characteristic structure with distinct hydrophobic
portions and positively charged amino acids and allow the formation of an amphiphilic conformation.

Figure 13. Secondary structures of AMPs: A) α-helical, B) β-turn/sheet, C) random coil, D) mixed α/β [55]

AMPs have been classified according to their specific properties in several groups: defensins, knottin-like, 2S albumins, cyclotides, lipid transfer proteins (LTPs), heveins and snakins.

10.3.12.1. Defensins

Defensins were the first studied AMPs, isolated from wheat and barley and named γ-thionins. Defensins have a low molecular weight of 5 kDa [53], a primary structure of 45–54 amino acids and have been isolated mainly from seeds, but also from other organs of the plant (leaves, stem, root, flowers). α- and β-thionins were discovered later. All three types of defensins have two or three disulfide bonds in the molecule, although their structures are different. Disulfide bonds play a role in stabilizing the structure of these peptides, consisting of a β-sheet conformation and an α-helical segment, so that the defensin molecules are particularly resistant to extreme temperature, pH and enzyme action.

Pelegrini et al., in 2008, isolated from Petunia hybrida two defensins, which they named PhD1 and PhD2, with different structures. Other defensins were found in Raphanus sativus, Vigna unguiculata, Fagopyrum esculentum Moench,
and *Phyllostachys pubescences*, that exhibit antibacterial and antifungal activity [53,55].

Plant defensins have demonstrated antifungal and antibacterial activity but can also act as enzyme inhibitors.

### 10.3.12.2. Knottin-like peptides

Knottin-like peptides were discovered in 1990 by Nguyen *et al.*, in *Ecballium elaterium* seeds, and were named knottins. Knottins contain about 40 amino acids and three disulfide bonds whose arrangement provide the structure with an extraordinary proteolytic, thermal and chemical stability [56]. Knottins possess cytotoxic, antimicrobial, insecticidal, anti-HIV and hormone-like activity. These compounds were isolated from *Mirabilis jalapa* and exhibit antifungal activity against 13 phytopathogenic fungi (*Botrytis cinerea*, *Alternaria brassicola*, *Fusarium oxysporum* and others) and antibacterial action against two Gram-positive bacteria (*Bacillus megaterium* and *Sarcina lutea*) [57]. Other knottin-like peptides were isolated from *Phytolacca americana* and had inhibitory action on *Alternaria tenuis*, *Fusarium graminearum*, *F. oxysporum* and *Trichoderma viridae* [58].

### 10.3.12.3. 2S Albumins

2S albumins are storage proteins with important plant survival functions, which also have a defensive role. They are water-soluble peptides, rich in basic amino acid glutamine, with a sedimentation coefficient of about 2 Svedberg units. They have a wide spread in monocotyledonous and dicotyledonous plants and were first discovered in castor seeds. They appear to be synthesized by post-translational modification processes by enzymatic cleavage. 2S albumins have different functions in the cell: protein storage, emulsion stabilization, acting as inhibitors of proteolytic enzymes and having antimicrobial activity. It has been shown that 2S albumins from *Arachis hypogaea* have antifungal action against *Aspergillus flavus* by inhibiting conidiospore germination. 2S albumins from *Passiflora edulis* had inhibitory action on the mold species of *Trichoderma harzianum*, *Fusarium oxysporum*, *Aspergillus fumigatus*. It was found that *Sesamum indicum* produces 2S albumines with antibacterial activity against *Klebsiella* sp. [53].

### 10.3.12.4. Cyclotides

Cyclotides are cyclic peptides formed consisting of 28–37 amino acids, found in some plants of the families *Rubiaceae*, *Violaceae*, *Poaceae* and *Fabaceae*. Cyclotides are synthesized by excisions from precursor polypeptides and cyclization. They are molecules with high resistance to extreme temperatures and chemical agents and have antimicrobial, antitumor, nematicidal, insecticidal, anti-HIV activity. Cyclotides synthesized in *Viola abissynica*, *V.
odorata and Oldenlandia affinis exhibit antibacterial activity against E. coli, Salmonella enterica and S. aureus [53].

10.3.12.5. Lipid transfer proteins (LPTs)

LPTs are small molecules of cationic peptides of between 7 and 10 kDa and have the ability to transfer lipids by reversibly binding and transporting them. LPTs have in the molecule eight cysteine residues with four disulfide bonds. These chemical bonds have the role of stabilizing the tertiary structure of four α-helices, which have a hydrophobic site where lipid molecules can bind. LPT classification is based on the differences between helix structures and their arrangement. It seems that LPTs play an important role in plant signaling, plant defense, and antimicrobial activities. LPTs have been found in Allium cepa, Helianthus anuus, Capsicum annuum, Raphanus sativus, Hordeum sp. and other plants [53,55].

10.3.12.6. Heveins

Heveins have been isolated from the first time by Archer in 1960 from the Hevea brasiliensis rubber tree. Heveins belong to the class of lectins and have the ability to chemically bind N-acetyl-glucosamine from the fungal cell wall. This peptide contains 43 amino acids and possesses similar structures to other lectins in plants. The hevein structure is composed of an anti-parallel β-sheet and a few short α-helices and is stabilized by three to five disulfide bonds. Due to their structure, heveins can bind chitin in the hyphal cell wall and thus stop the apical growth of the fungi. However, it has been observed that heveins can inhibit the growth of chitin-free microorganisms such as oomycetes and bacteria [53]. Heveins have been isolated from Pharbitis nil and Triticum kiharae.

10.3.12.7. Snakin / Gasa peptides

Snakins are peptide molecules rich in cysteine (19 %), with two to five disulfide bonds, composed of about 63 amino acid residues [53]. The primary structure of snakins is similar to the snake venom hemotoxic protein. To date, no tertiary structure has been discovered for these peptides. Porto et al. proposed a structure containing six disulfide bonds formed from three helices.

A quite different class of peptides are the short non-disulfide peptides, called so because they are cysteine-free or present very low cysteine content. An example of this group of peptides is ginkbilobin from Ginkgo biloba, consisting of 40 amino acids, with antimicrobial effect against the bacterial species of S. aureus, P. aeruginosa and E. coli and several fungi. Two cysteine-free AMPs were isolated from Capsella bursa-pastoris and were named sheeperin I, and sheeperin II that were active against E. coli, Pseudomonas putida, P. syringae and Serratia sp. and against the fungi C. albicans, Cryptococcus neoformans and Saccharomyces cerevisiae, respectively [51].
Other peptides in plants with antimicrobial activity are myrosinase binding protein, glycine-rich protein, $\alpha$-hairpins, $\alpha \beta$-trumpet, and others.

Because the AMPs in plants can only be obtained in small amounts, the isolation of AMPs from a natural source is not economically sustainable. Thus, it is necessary to find different methods for producing AMPs. These methods refer to chemical synthesis or DNA recombinant technology. In the chemical synthesis the costs are also high for the production of sequences longer than ten amino acid residues, and costs are rising more for the production of specific disulfide bridges. Recombinant DNA technology facilitates the production of AMPs on a large scale by cloning the genes of interest in vectors for expression in host cells [53].

10.4. MECHANISM OF ANTIMICROBIAL ACTION

Different modes of functions have been proposed for the antimicrobial compounds as a whole. It is obvious that one solitary mechanism has a weaker effect against a microbial cell, but an assembly of mechanisms of antimicrobial functions have a strong impact on cellular structures. In general, plants synthesize complexes of related or chemically different secondary metabolites, the action of which could occur synergistically. In addition, the effect of plant metabolites can be emphasized in the presence of traditional antibiotics [60].

Several major mechanisms (Table 1) of antimicrobial activities of plant extract are described by Djilani and Dicko [61]:

1) Alteration of cytoplasmic membrane structures.
2) Interaction with extracellular proteins (for example ATPase).
3) Disturbance and inactivation of the function of the outer membrane in Gram-negative bacteria by modifying lipopolysaccharides.
4) Fluctuation of the proton engine force of the cells with permeance of ions.
5) Coagulation of cytoplasmic contents.
6) Prevention of enzyme generation.
<table>
<thead>
<tr>
<th>Class</th>
<th>Subclass</th>
<th>Examples</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolics</td>
<td>Simple phenols</td>
<td>Catechol</td>
<td>Substrate deprivation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epicatechin</td>
<td>Membrane disruption</td>
</tr>
<tr>
<td>Phenolic acids</td>
<td>Cinnamic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quinones</td>
<td>Hypericin</td>
<td></td>
<td>Bind to adhesins, complex with cell wall, inactivate enzymes</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Chrysin</td>
<td></td>
<td>Bind to adhesins</td>
</tr>
<tr>
<td>Flavones</td>
<td>Abyssinin</td>
<td></td>
<td>Complex with cell wall, inactivate enzymes, inhibit HIV reverse transcriptase</td>
</tr>
<tr>
<td>Flavonols</td>
<td>Totarol</td>
<td></td>
<td>Not identified</td>
</tr>
<tr>
<td>Tannins</td>
<td>Ellagittannin</td>
<td></td>
<td>Bind to proteins, bind to adhesins, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation</td>
</tr>
<tr>
<td>Coumarins</td>
<td>Warfarin</td>
<td></td>
<td>Interaction with eucariotic DNA (antiviral activity)</td>
</tr>
<tr>
<td>Terpenoids, essential oils</td>
<td>Capsaicin</td>
<td></td>
<td>Membrane disruption</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>Berberine</td>
<td></td>
<td>Intercalate into cell wall and/or DNA</td>
</tr>
<tr>
<td></td>
<td>Piperine</td>
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<tr>
<td>Lectins and polypeptides</td>
<td>Mannose-specific agglutinin</td>
<td>Fabatin</td>
<td>Block viral fusion or adsorption Form disulfide bridges</td>
</tr>
<tr>
<td>Polyacetylenes</td>
<td>8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol</td>
<td></td>
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</tr>
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</table>

The study on the interactions of these compounds with the microbial cell demonstrated that the major cellular targets include (Figure 14) [59]:

1. The biomembrane;
2. Proteins (cell receptors, ion channels, enzymes, transport systems, regulatory proteins, structure proteins, transcription factors);
3. Nucleic acids (DNA and RNA).
1. The biomembrane structure is deteriorated by lipophilic or amphiphilic molecules present mainly in terpenoids. These hydrophobic compounds alter the double layer of phospholipids in membranes forming transient pores that modify the permeability of the cell envelope and allow the diffusion or transport of harmful compounds. Other secondary metabolites can interact with the extramembrane or transmembrane proteins (ion channels, transporters, receptors), blocking their function of signaling and transport. This effect can be transient or permanent and leading to cell death.

2. Antimicrobial metabolites can interact with cell proteins, thus altering the secondary and tertiary structure of these important molecules. When the three-dimensional conformation of proteins is changed, their structural and metabolic functions are modified with dramatic consequences in cell life. The conformational changes can either activate or inactivate a protein. The secondary metabolites can also modify the conformation of the catalytic center of enzymes causing the loss of catalytic function. The correct conformation is indispensable for the recognition of the substrates, ligands and other substances. Some highly reactive antimicrobial compounds can make covalent bonds with proteins, and others may form physical bonds such as hydrogen bonds, dipol-dipol or others. The most reactive metabolites belong to the classes of aldehydes, epoxides, sulfhydryls, exocyclic methylenes or cyclopropanes [59]. The hydroxyl groups of phenolic compounds can dissociate under physiological conditions into negatively charged O-groups. These negative groups can react with the basic amino acid residues in proteins thus modifying the three-dimensional conformation.

3. Nucleic acids. Secondary metabolites containing alkyl groups or molecules similar to nitrogenous bases in DNA or RNA can modify nucleic acids by alkylation and intercalation. These changes can lead to point mutations, and finally the primary sequences of amino acids in proteins are modified, if the

---

**Figure 14.** Target bacterial structures for antimicrobial compounds [59]
mutations are not repaired. In fact, antimicrobial metabolites can affect the whole replication, transcription and translation systems [59].

10.5. SYNERGISTIC ACTION OF PLANT METABOLITES AND ANTIBIOTICS

A relatively new trend in the development of new sources of antibiotics is the study of the combination between natural plant derivates and standard antibiotics in order to enhance their activity through bactericidal synergism. For example, the pluripotent activity of phytochemicals may stimulate the antimicrobial activity of aminoglycosides, quinolones, macrolides, and tetracyclines. In multidrug therapy, the obtained effects may be insignificant (when the effect of two compounds is the same), additive (as the sum of effects), synergistic, or antagonistic. In the case of a synergistic effect, the activity of the combination of compounds is higher than the sum of the effects of individual compounds [3].

It was demonstrated that extracts of *Punica granatum* in combination with chloramphenicol, ampicillin, gentamicin, tetracycline, and oxacillin have a synergistic effect on MRSA bacteria. The berberine alkaloid berberine exhibits a highly synergistic activity with β-lactam antibiotics against MRSA [3].

10.6. CONCLUSIONS

Since ancient times, plants have traditionally been used for the prevention and treatment of various diseases. Plants represent a promising alternative to treating medically challenging pathogens and to combat the growing number of bacteria that have become resistant to conventional antibiotics. In addition, with the increased negative attitudes of consumers to chemical preservatives, the use of antimicrobial plant extracts has become an especially interesting alternative.

Plant cells produce a variety of phytochemicals, especially secondary metabolites for defense mechanisms against microorganisms, parasites, and herbivores. These bioactive compounds are present in all plant material (roots, stems, leaves, flowers, fruits, seeds) and are responsible for the medicinal properties and health benefits of herbs. These metabolites belong to a large number of classes of chemical compounds: phenols, alkaloids, quinones, terpenes, essential oils, tannins, saponins, glycosides, lignans, and peptides, whose identification and isolation is still a challenge.
The structure of antimicrobial secondary metabolites explains the variety of mechanisms by which these compounds act against microbial cells, different cellular architectures and target functional groups. Studies on the interactions of these compounds with the microbial cell have demonstrated that the major cellular targets include the biomembrane, proteins (cell receptors, ion channels, enzymes) and nucleic acids (DNA and RNA). Several mechanisms of action of antimicrobial metabolites in plants consist of alteration of membrane structures, interaction with extracellular proteins, fluctuation of the proton engine force of the cells, and coagulation of cytoplasmic contents.

Antimicrobial compounds in plants as a whole have a broad spectrum of action that can exceed the limited specificity of antibiotics. In this context, a relatively new trend in the development of new sources of antibiotics is the study of combination between natural plant derivates and standard antibiotics, in order to enhance the activity of latter through bactericidal synergism.

REFERENCES

Chapter 11

EQUIBIOTICS: A NEW TYPE OF PHYTO-DRUG EQUILIBRATING LOCAL MICROBIOTA

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11.1. THE HUMAN MICROBIOME

During the last decades of the twentieth century, the evolution of knowledge on microbial genetics has revealed the existence of a permanent and indispensable functional relationship between microbes of the natural environment and cells of certain tissues of the human body. The first discoveries made about microbes normally present in some tissues of the healthy human being, revealed the role played by what were then called 'intestinal flora' on the digestive function of the large intestine. Subsequent studies led to the discovery and understanding of the existence of a large set of families of microorganisms whose presence was indispensable for the proper functioning of the digestive tract. Later, the confirmed presence of millions of different non-pathogenic microorganisms in the skin, the respiratory and genitourinary tracts came to be, in classic microbiology, interpreted as 'symbiotic' and benefiting the healthy 'host'.

Today the understanding of such important microbe/cell interactions has evolved. The studies and techniques used in the discovery of the human genome applied to the genetic content of the microbial collection permanently lodged in cell tissues of all animals now is considered the microbiome.

According to its definition, the human microbiome is the genetic aggregate of microorganisms that reside on or within any of a number of human tissues and biofluids, including the skin, mammary glands, placenta, seminal fluid, uterus, ovarian follicles, lungs, saliva, oral mucosa, conjunctiva, biliary and the mucous membrane of respiratory and gastrointestinal tracts. They include thousands of bacteria, archaea, fungi and viruses and differ in each person. The term 'human microbiome' refers specifically to the collective genomes of resident microorganisms considered a counterpart to the human genome. The study of the 'communication' of these two great genetic systems results in the key to understand the functioning of the human body [1-3].

Consequently, in this field of research, an ecosystem is defined as the functional unit composed of the local set of microbes (microbiota) and the set of human cells (local tissue) that share a habitat (specific physic-chemical local conditions) that guarantee their interaction. For example, in the ecosystem of the digestive tract, microorganisms find the components they require for their survival and proliferation on the surface of the mucosa, giving rise to the formation of specific microbiota (buccal, oesophageal, gastric, pyloric, duodenal, colonic, rectal, etc.). By finding the right local conditions, the different microbial colonies reproduce and synthesize bioactive compounds (messenger molecules that monitor processes, activate immunological responses, trigger genetic functions, etc.) which, once incorporated into the bloodstream by cells, act remotely on other organs of the human body [4-6].
In all these ecosystems, colonies of microbes vary in density (quantity) and diversity (types of families and genera). Depending on the local physicochemical conditions, a state of biological balance or "equilibrium" with the human cells develops at the site. The newest interpretations of this extraordinary phenomenon have allowed us to extend the old concept of homeostasis or 'physiological self-regulation' of the human body. Homeostasis is now considered the result of genetic functional interaction between both the human microbiome and genome. This scope modifies the medical interpretation of the binomial health-disease paradigm [7].

Thus, the origin of infectious or pathogenic microbial colonization processes has to be considered as a rupture of the equilibrium of the microbiota/tissue relationship, a condition called dysbiosis.

In accordance with this scope, the strategy to treat common infectious diseases is now different from that practiced by medicine for most of the 20th century. The previous medical paradigm of maintaining the human body in almost aseptic conditions through the systematic annihilation of pathogenic microbes with potent antibiotics and antiseptics of all kinds has often been counterproductive. The worldwide re-emergence of infectious diseases previously controlled by antibiotics now rendered ineffective is due to the formation of "resistant" microbial strains to such drugs and the emergence of genetic variants of previously non-existent pathogens. In addition, environmental air and water pollutions, chemical composition of modified animal and vegetable foods, among many other factors, have contributed to the origin of multiple functional alterations of the human microbiome [8].

Some of these microbiome alterations are manifested as metabolic disorders (diabetes, obesity, malnutrition) or as central nervous system dysfunctions (insomnia, Alzheimer's disease, infantile dementia), or as altered autonomic manifestations (respiratory and cardiac dysrhythmias, fibrosis, asthma) or by disruptions in the immune system (immuno-deficiencies, allergies), etc. [9-11].

Dysbiosis of the digestive tract microbiota will give rise to many conditions now considered part of the same process. In the mouth, alteration of the local microbiota will produce dental caries and periodontitis; in the stomach, reflux, gastritis, stomach ulcers, dyspepsia; in the intestine, conditions such as irritable bowel syndrome, chronic diarrhoea or constipation, malabsorption, diverticulitis, etc. [12-14].

In the search for solutions to intestinal dysbiosis, the so-called prebiotics (specific nutrients for microbes) have gained great importance as useful functional foods that benefit the organism by stimulating the growth and activity of one or more strains of 'benign' bacteria present in the intestine. Similarly, the probiotics are foods added with living specific microorganisms that remain active in the intestine and have important effects on the constitution of the appropriate microbiota of the digestive tract [15,16].
11.2. THE EQUIBIOTICS

In the last two decades, the renaissance of phytotherapy research all around the world has boosted the scientific study of medicinal plants in many countries. Medicinal plants selected from the so-called ‘traditional herbal medicines’ used historically by different medical cultures are now scientifically studied in the search for alternative products for the treatment of common infections. The existence of plant remedies with attributed ‘antimicrobial’ properties represents a huge chapter of herbal medicine in many places [17-20].

In Mexico, researchers from our centre have worked in this direction developing phytodrugs made from medicinal plant extracts popularly used to attend to common infectious ailments. The results of this research allowed the designing of a type of plant extract mixture, characterized as ‘equibiotics’ acting on the maintenance or restoration of the microbiome balance.

According to its definition, equibiotics act simultaneously in two modalities: with auferobiotic and alerebiotic effects. The first refers to the ability of some plant extracts to avoid the proliferation of certain pathogenic microbes and, the second, their complementary capacity to restore conditions that favour the normal balance of a local microbiota [21-23].

The auferobiotic effect is a concept related to several mechanisms of growth inhibition of pathogenic microbes induced by certain bioactive plant compounds. According to the literature, several medicinal plant extracts, considered as ‘antimicrobial’ agents, possess a capacity not necessarily based on a destructive effect on the pathogen strain. For example, they may inhibit the proliferation of pathogens by a bacteriostatic effect, or paralyzing and impeding the adhesion of certain bacteria to the mucous membranes, or stimulating the production of specific bacteriocins in other ‘positive’ strains of the ecosystem, etc.

On the other hand, the alerebiotic effect observed during the use of equibiotics is manifested by favouring reconstruction of local tissues, balancing oxidative processes, reducing inflammation, reinforcing local immune responses, or interacting with bacterial genes and inducing proliferation of ‘beneficial’ strains [24,25].

During the last years, we have been performing studies for the design and use of these kinds of plant extracts. As an example, we describe the characteristics of an equibiotic plant product proposed to balance the microbiota in the digestive tract.
11.3. EQUIBIOTIC-GI (GASTRO-INTESTINAL)

The herbal medicine named Equibiotic-GI, developed for the treatment of chronic dyspepsia, whose more common symptoms are stomach distension and pain, gastritis, nausea, abdominal sensation of fullness, flatulence and chronic colitis with alternative episodes of diarrhoea or constipation [26].

Equibiotic-GI contains two extracts. One is obtained from the leaves of Psidium guajava L. (Myrtaceae) and the second, from the roots of Coptis chinensis Franch. (Racunculaceae). Both plants are used in herbal medicines in several countries of the Americas and Asia, with pharmacological properties widely recognized as anti-microbial, anti-diarrheal, anti-oxidant, spasmolytic, digestive, anti-diabetic and sedative (Figures 1 and 2).

![Psidium guajava](image1.png)

**Figure 1.** *Psidium guajava* L. (Fam. *Myrtaceae*) popularly named Guava; the leaves are used in Mexican traditional medicine in the concoction of tisanes for the treatment of gastrointestinal disorders such as diarrhoea, chronic colitis, gastritis, etc.

*Psidium guajava* is a species originally from Mexico and Central America and its leaf extracts have been extensively reported in modern scientific literature due to its content of derivatives from flavonoids quercetin and morin, considered bioactive [27].
Equibiotics: a new type of phyto-drug equilibrating local microbiota

Figure 2. *Coptis chinensis* Franch. (Fam. *Racunculaceae*) popularly named Huang Lian. According to traditional Chinese medicine, the root is a pungent, very bitter, cooling herb that controls bacterial and viral infections, relaxes spasms, lowers fevers and stimulates the circulation.

*Coptis chinensis* root extract is also widely used in Asia for the treatment of gastrointestinal infections, chronic bowel syndrome and diverse digestive ailments, and its bioactivity related to the content in alkaloidal berberine derivatives [28].

Equibiotic-GI is a phytodrug composed of a dry extract of *Statum coptis chinensis*, standardized in its content of isoquinoline alkaloids, quantified as berberine (≥ 6.0 %) and a hydro-glycolic extract of *Folia psidii guajavae*, standardized in its content of flavonic glycosides, quantified as quercetin (≥ 0.1 %). The mixture is pharmaceutically formulated as a suspension for oral administration, for a ten day treatment to alleviate chronic dyspepsia.

Due to the fact that *Helicobacter pylori* invasion is generally associated with chronic gastritis and, in general, to dyspepsia syndrome, initially the studies that allowed evaluation of the properties of this combination of extracts, were performed with in vitro cultures of several clinically obtained strains of the bacterium *H. pylori*, resistant to conventional antibiotics.

Both extracts inhibited the proliferation of *H. pylori* antibiotic-resistant strains. Minimum inhibitory concentration (MIC) obtained with the mixture of extracts to produce 100 % inhibition of the growth of the bacteria cultures, showed a remarkable synergistic ability to reduce the MIC value by 500 and 1000 times, compared with the test of both extracts separately. The following studies determined the property of the mixture to prevent the adhesion (local and diffuse) of the *Helicobacter* to gastric epithelial cells (AGS) cultivated in vitro [29].
To these multiple *in vitro* bioactive effects of the plant extract mixture on *H. pylori*, followed conventional preclinical toxicological studies of the pharmaceutical form developed as Equibiotic-GI for oral human administration [30].

Once the non-toxic effects of the product were established, the clinical trial consisted of a Phase III study in a group of volunteers (*n* = 30) with chronic functional dyspepsia. The study included the DNA analysis of the intestinal microbiota of the patients before and after administering the Equibiotic-GI. The treatment was given to the participants as home treatment (20 mL of suspension, once daily, v.o., during 15 days) maintaining the habitual diet and life style.

At the beginning of the study, a medical interview assessed the clinical history and determined signs and symptoms of functional dyspepsia in every participant. A faecal sample was collected before starting the treatment. In a second session after treatment, a medical interview was performed to determine clinical changes observed and the corresponding faecal sample obtained. Samples from each participant, before and after the study, were processed using conventional methods established to obtain DNA of the intestinal microbiota, determining two levels of genetic identification, by microbial families and by genera [31].

Results obtained showed improvement of all dyspepsia symptoms: drastic disappearance of abdominal pain, reduction of intestinal inflammation and flatulence, marked reduction of episodes of gastritis, disappearance of nausea, increase of appetite and improvement of quality of sleep and mood.

The micro-genomic studies showed a significant increase in the *Bacteriodetes* families and a moderate reduction in the *Firmicutes* families, detected in all cases after treatment. In subsequent genera and species analysis, the intestinal microbiota post-treatment changes were qualified as proliferation of positive microbial strains, together with a notable increase in diversity (Figures 3 and 4).

The increase of 'positive genera' was interpreted as manifestations of recovery of the microbiotic normal balance in each individual and, in all cases, included a greater diversity of strains and a significant reduction of groups considered 'potentially pathogenic'. Finally, a predominance of microbial species linked to the reduction of intestinal inflammation and to positive immuno-protective responses against possible pathogens were confirmed in all cases.
Equibiotics: a new type of phyto-drug equilibrating local microbiota

**Figure 3.** Intestinal microbiograms of three patients (P2, P15, P23) with gastrointestinal dysbiosis, before and after treatment with Equibiotic-GI. Note the increment of *Bacteriodetes* genera in all cases.

**Figure 4.** Drawing that illustrates the mucosa of the intestine before and after administration of Equibiotic-GI.
11.4. CONCLUSIONS

The extract obtained from *Psidium* is particularly rich in flavonoids; the most abundant includes quercetin, morin, kamferol, rutin and guajaverin. All of these compounds have recognized anti-microbial activity against very diverse bacterial strains that cause important infections. According to our observations, the nature of this anti-microbial activity is predominantly bacteriostatic, affecting adhesion and not necessarily destroying the strain [32,33]. Nevertheless, flavonoids involved in anti-microbial actions may use the following mechanisms:

Inhibition of membrane function affecting its permeability and causing the disruption of its barrier function. This produces the interruption of the normal flow of nutrients, while inducing the loss of essential components for the development and proliferation of the microorganism. With this effect, the motility, chemotaxis, adhesion and invasive capacity of the bacteria should be altered.

Inhibition of the synthesis of nucleic acids (DNA and RNA) through the intercalation of hydrogen bonds in the structure of the nitrogenous bases that form them, and through inhibition of enzymes involved in the synthesis processes of the DNA and RNA of microorganisms.

Inhibition of energy metabolism, acting on the respiratory chain and bacterial electronic transport, affecting the synthesis processes of macromolecules essential for the development of the bacteria. All of these processes can occur simultaneously, depending on the dose and combination of flavonoids used.

Additionally, we add the fact that according to the scientific literature, other plant substrates with recognized anti-microbial properties have been reported and contain alkaloid-type active ingredients, particularly those rich in hydrophobic cations, which attack the membrane itself and the bacterial DNA. Such is the case of the extracts of plants of the genus *Coptis* because they contain isoquinolines and many other abundant compounds structurally related to berberine, a hydrophobic cation that easily crosses the microbial membrane and that accumulates inside the bacterium, taking advantage of the alterations it causes in its potential trans-membrane. This property of accumulation of the berberine derivatives inside bacteria is more than 1000 times higher than that of other molecules of plant origin, which makes them essential assets in the sought after anti-microbial effect.

*Coptis* extract also contains 5’-methoxyhydnocarpine (5’-MHC), a powerful inhibitor of bacteria efflux pumps, which potentiates the antimicrobial action of berberine at very low concentrations. It is interesting to mention that the 5’-MHC has a high chemical structural analogy with the flavonoid quercetin and that its inhibitory capacity for microbial growth related to the glycoproteins that operate the microbial efflux pumps (MDRs).
In addition, everything seems to indicate that the flavonoid and alkaloidal effects are also related to their ability to interfere with efflux pumps and the mechanism of "quorum sensing" (QS) of undesirable bacteria in the ecosystem, a complex mechanism of action yet to be tested experimentally [34-38].

Based on the above, data indicates that the extracts of *Psidium guajava* and of *Coptis chinensis*, mixed in a range of synergistic proportions, owe their peculiar auferobiotic effect mainly to the combination of the quercetin and berberine derivatives [39,40].

On the other hand, the alerebiotic effect of the phytodrug Equibiotic-GI, was verified by the positive changes provoked in the composition of the gut microbiota of patients with functional dyspepsia. The observation also corroborates the relationship that exists between symptomatic relief and therapeutic benefits with the configuration of the microbiota.

In this sense, we demonstrated the so-called equibiotic effect by corroborating that a state of "normal" or "in equilibrium" intestinal microbiota can be induced. However, the complex mechanisms that are involved in this phenomenon need still to be deeply investigated.

**ACKNOWLEDGMENTS**

The clinical studies for the development and assessment of the Equibiotic-GI were carried out in the Infectious Diseases Research Unit of the Mexican Institute of Social Security (IMSS), in the Preclinical Research Unit (UNIPREC) of the National Autonomous University of Mexico (UNAM) and in the National Institute of Genomic Medicine (INMEGEN).

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Chapter 12

VETERINARY ANTIMICROBIAL STEWARDSHIP

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Chapter 12

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12.1. USE OF ANTIMICROBIALS IN VETERINARY MEDICINE AND THE EMERGENCE OF RESISTANCE

12.1.1. Introduction

Antimicrobials have played a vital role in human and animal health care for more than 50 years. Antimicrobial resistance (AMR) has escalated rapidly to become a major public health crisis globally. AMR is now a major threat to human and animal health and is placed in the same risk category as climate change and overpopulation. Antimicrobials are important in animal health for the treatment and/or prevention (prophylaxis) of disease in both small companion animals and food animals and also in metaphylaxis and growth promotion in food animals for a sustainable and economically viable animal industry [1]. The various uses of antibiotics in animal health are depicted diagrammatically in Figure 1.

Figure 1. The use of antibiotics in animal health [2]
12.1.2. Emergence of resistance in animal health

However, the overuse and inappropriate use of antimicrobials has resulted in the selection of resistant bacterial populations, as bacteria try to adjust and survive in the unfavourable antimicrobial conditions. Other contributing factors to increasing AMR are:

- Socio-economic disparity with concomitant sanitation issues in many parts of the world
- Poor prescription and dispensing practices
- Lack of national antibiotic resistance surveillance networks
- Inadequate infection control in many hospitals/animal facilities
- The use of antibiotics in feed and water of production animals [2].

Resistant bacteria selected through injudicious use of antimicrobials in companion animals can have a double impact of first making future treatment ineffective as well as having the potential to spread to people through the exchange of resistance genes with bacteria resident in or on the human host. In food animal production, animal bacteria, including resistant strains and/or their resistance determinants may spread to humans by direct contact, through the food chain and by environmental contamination; the two former routes being of major importance [2]. AMR through the food chain may be transferred to humans in the following ways:

- Antibiotic-resistant bacteria pathogenic to humans are selected and the foodstuff becomes contaminated with these bacteria during slaughter and/or the food preparation process. This foodstuff is then ingested and causes an infection for which antibiotic treatment is required and which is ineffective as a result of the antibiotic resistance.
- Antibiotic-resistant bacteria non-pathogenic to humans are selected in the animal and the foodstuff becomes contaminated with these bacteria during slaughter and/or the food preparation process. This foodstuff is then ingested and the bacteria transfer the resistance to other bacteria in the human gut.
- Antibiotics remain as residues in the food animal products which then allows for the selection of antibiotic resistant bacteria in gut of the consumer of this food [1].

Typical zoonotic pathogens include *Salmonella* spp., *Campylobacter* spp. and shiga-toxin producing *E. coli* strains. However, many other bacteria are known to spread from animals to humans including other *E. coli* strains, *Yersinia enterocolitica* and *enterococci* [1]. Still, the link between use and overuse of antibiotics and resistance is not easy to follow, as AMR is a very complex and non-victimless phenomenon affecting both human and animal health [3].
Figure 2 shows the complex inter-relationship between antibiotic use in humans, animals and the environment.

12.1.3. Current trends of veterinary antimicrobial use in food-producing animals

The use of veterinary antimicrobial agents in food-producing animals in countries of European Union and the United States of America is presented in Table 1 and Table 2, as reported by the European Medicines Agency, and the US Food and Drug Administration (FDA). According to these data, the consumption of antibiotics for animal use has been augmented by about 4% in the European Union, whereas in the United States, it has followed an ascending trend, despite the call for limiting antimicrobial use in livestock. The FDA however amended the new animal drug regulations in 2015 to implement the veterinary feed directive (VFD) drugs section of the Animal Drug Availability Act of 1996 (ADAA) as from 1 October 2015 which rules the following: A VFD drug is intended for use in animal feeds, and such use of the VFD drug is permitted only under the professional supervision of a licensed veterinarian [3].
Table 1. Sales (tons of active ingredient) of veterinary antimicrobial agents applicable mainly for food-producing animals, including horses, on average per European Union member state between 2005 and 2012 [3]

<table>
<thead>
<tr>
<th>Antimicrobial class</th>
<th>2005</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetracyclines</td>
<td>148.6</td>
<td>143.7</td>
<td>157.4</td>
<td>134.4</td>
<td>119.0</td>
<td>123.2</td>
<td>113.2</td>
</tr>
<tr>
<td>Amphenicols</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>1.1</td>
<td>1.1</td>
<td>2.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Penicillins</td>
<td>34.7</td>
<td>35.7</td>
<td>35.0</td>
<td>35.7</td>
<td>37.1</td>
<td>77.9</td>
<td>68.5</td>
</tr>
<tr>
<td>Cephalosporins (total)</td>
<td>1.7</td>
<td>1.9</td>
<td>1.9</td>
<td>1.7</td>
<td>0.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>First- and second-generation cephalosporins</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.1</td>
<td>1.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Third- and fourth-generation cephalosporins</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Sulfonamides and trimethoprim (total)</td>
<td>53.7</td>
<td>53.3</td>
<td>56.6</td>
<td>51.6</td>
<td>48.7</td>
<td>72.6</td>
<td>36.7</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>46.0</td>
<td>45.7</td>
<td>48.9</td>
<td>44.6</td>
<td>42.0</td>
<td>36.3</td>
<td>31.8</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>7.7</td>
<td>7.6</td>
<td>7.7</td>
<td>7.0</td>
<td>6.7</td>
<td>5.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Macrolides</td>
<td>21.3</td>
<td>24.0</td>
<td>24.4</td>
<td>23.3</td>
<td>21.0</td>
<td>27.2</td>
<td>24.5</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>2.3</td>
<td>2.0</td>
<td>2.0</td>
<td>1.7</td>
<td>1.7</td>
<td>9.7</td>
<td>9.0</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>14.6</td>
<td>14.4</td>
<td>13.9</td>
<td>13.0</td>
<td>13.0</td>
<td>6.6</td>
<td>11.2</td>
</tr>
<tr>
<td>Quinolones (total)</td>
<td>4.6</td>
<td>4.6</td>
<td>4.4</td>
<td>4.0</td>
<td>3.3</td>
<td>10.9</td>
<td>7.2</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>0.9</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
<td>5.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Other quinolones</td>
<td>3.7</td>
<td>3.6</td>
<td>3.4</td>
<td>2.9</td>
<td>2.1</td>
<td>2.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Polymyxins</td>
<td>10.0</td>
<td>10.1</td>
<td>11.3</td>
<td>10.1</td>
<td>10.3</td>
<td>22.4</td>
<td>21.0</td>
</tr>
<tr>
<td>Pleuromutilins</td>
<td>2.7</td>
<td>2.6</td>
<td>3.0</td>
<td>3.6</td>
<td>3.6</td>
<td>8.5</td>
<td>8.8</td>
</tr>
<tr>
<td>Others:</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>9.0</td>
<td>3.9</td>
</tr>
<tr>
<td>Total</td>
<td>295.6</td>
<td>293.7</td>
<td>311.6</td>
<td>280.9</td>
<td>261.1</td>
<td>336.8</td>
<td>307.0</td>
</tr>
</tbody>
</table>

Notes:
*Others include bacitracin, paromycin, spectinomycin, polymyxins, and amphenicols. Data were derived for cumulative reports involving seven European Union member states in 2005–2009, 25 in 2011, and 26 in 2012. Data from the European Medicines Agency.
Table 2. Antimicrobial drugs approved for use in food-producing animals actively marketed in the United States between 2009 and 2012 (tons of active ingredient) [3]

<table>
<thead>
<tr>
<th>Drug class</th>
<th>2009</th>
<th>2010</th>
<th>2011</th>
<th>2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>223.12</td>
<td>211.79</td>
<td>214.89</td>
<td>273.53</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>20.14</td>
<td>24.59</td>
<td>26.61</td>
<td>27.65</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>93.33</td>
<td>154.65</td>
<td>190.1</td>
<td>218.14</td>
</tr>
<tr>
<td>Macrolides</td>
<td>562.06</td>
<td>553.23</td>
<td>582.84</td>
<td>616.27</td>
</tr>
<tr>
<td>Penicillins</td>
<td>691.64</td>
<td>884.42</td>
<td>885.3</td>
<td>965.2</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>505.9</td>
<td>517.13</td>
<td>383.1</td>
<td>493.51</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>5,260.99</td>
<td>5,602.28</td>
<td>5,652.85</td>
<td>5,954.36</td>
</tr>
<tr>
<td>Ionophores</td>
<td>3,739.35</td>
<td>3,820</td>
<td>4,122.4</td>
<td>4,573.79</td>
</tr>
<tr>
<td>Not independently reported (medically important)</td>
<td>329.39</td>
<td>281.22</td>
<td>319.99</td>
<td>344.43</td>
</tr>
<tr>
<td>Not independently reported (not medically important)</td>
<td>1,161.54</td>
<td>1,237.78</td>
<td>1,190.94</td>
<td>1,151.53</td>
</tr>
<tr>
<td>Total</td>
<td>14,618.43</td>
<td>12,587.46</td>
<td>13,287.1</td>
<td>13,569.04</td>
</tr>
</tbody>
</table>

Notes:
* Amphenicols, diaminopyrimidines, fluoroquinolones, and streptogramins;
† Aminocoumarins, glycolipids, pleuromutilins, polypeptides, and quinoxalines. Data from the FDA Center for Veterinary Medicine.
Abbreviation: FDA, US Food and Drug Administration.

In cattle, antimicrobials such as amoxicillin, penicillin, erythromycin, quinolones, gentamicin, novobiocin, tylosin, tilmicosin, and tetracycline are extensively used. In meat-producing animals, antibiotics are mainly used for the treatment and prevention of bovine pneumonia, diarrhea, and shipping fever, which are the most common problems. For the treatment of pneumonia, oxytetracyclines and spectinomycin are the first-choice antibiotics, with florfenicol and macrolides (particularly tilmicosin) considered as the second choice, with second-, third-, and fourth-generation cephalosporins being the last choice. Still, antibiotics are administered at least once via feed for various reasons, such as liver abscesses, increased growth, and respiratory diseases. The use of narrow-spectrum antimicrobials is favored in cases of clinical mastitis, with first-choice antimicrobials being the β-lactam antimicrobials used when treating mastitis resulting from streptococci, or penicillin when treating mastitis caused by staphylococci. In certain cases, the use of
antibiotics intramammary in the non-lactating period is given to the whole herd to prevent infectious mastitis [3].

In pigs, the current trends in husbandry require animal segregation in groups according to age, where pigs are of similar size and weight, and therefore the antimicrobials can be administered in groups of pigs via the oral route by addition in the feed or water. Individual therapy of pigs by injection of antimicrobials is mainly considered in pigs reared for reproduction. Use of antimicrobials for prevention is a common practice in pig farms, especially in stressful periods that predispose for infectious diseases. Such periods are the time between birth and first lactation, where the cut of the umbilical cord and tail and the trimming of the canines takes place; the ablactation period, where the environment and diet change and the castration of males and vaccinations take place; and finally the fattening period, where overcrowding, inadequate aeration, and low or high temperatures can form a quite stressful environment. Prophylactic use of antimicrobials is considered to be higher in the ablactation period, whereas at the end of fattening pigs, they do not receive antimicrobials so as to avoid residues detection after slaughter. For the prevention and treatment of enzootic pneumonia, large quantities of various antibiotics are used, with the most common being ceftiofur, tetracyclines, tiamulin, lincomycin, and enrofloxacin. In addition, in bacterial enteritis, especially when the etiological agent is *E. coli* or *Clostridium perfringens*, antibiotic treatment with penicillins, tetracyclines (chlortetracycline, oxytetracycline), quinolones (enrofloxacin), or aminoglycosides (gentamicin, neomycin) is required. Finally, in swine dysentery (*Brachyspira hyodysenteriae*), lincomycin, tiamulin, macrolides, or tetracyclines are mainly used [3].

In poultry, antibiotics used for therapeutic reasons are usually administered through water, in contrast to growth-promoting use, where antibiotics are added in feed. The most commonly used antibiotics are penicillins (amoxicillin), quinolones (enrofloxacin), tetracyclines (doxycycline, oxytetracycline), macrolides (erythromycin, tylosin), aminoglycosides, the sulfonamide/trimethoprim combination, polymyxins (colistin), and other antimicrobials (tiamulin). In the United States, the above mentioned antibiotics are used, with the exception of fluoroquinolones [3].

The antimicrobials commonly used in sheep and goats are amoxicillin, ampicillin, ceftiofur, the combination of amoxicillin/clavulanic acid, enrofloxacin, erythromycin, lincomycin, oxytetracycline, sulfonamides, penicillin G, trimethoprim and sulfonamide combination, tylosin, and tilmicosin (with the exception of goats, where subcutaneous injection of tilmicosin has been linked to death). Ampicillin, erythromycin, lincomycin, the trimethoprim and sulfonamide combination, and certain sulfonamides (*e.g.*, sulfathiazole) can significantly alter the microbial flora of the rumen when administered per os, and in certain cases, they can lead to death. Therefore, in the mature small ruminants, it is preferable to administer antimicrobials in
other ways than the oral route (feed or water), with the exception of certain sulfonamides and tetracyclines, which can be absorbed efficiently by the rumen [3].

Concerns about the extensive use of nontherapeutic agents have arisen after the duplication of the antimicrobial use in aquaculture in the decade 1994–2004. In aquaculture animals, several classes of antibiotics have been used. Among them are antibiotics such as sulfonamides, penicillins, quinolones, tetracyclines, and phenicols, which are listed as critically or highly important antimicrobials for human medicine. The last three antimicrobial classes are widely used in salmon farming. Quinolones, tetracyclines, and phenicols are selective for a variety of AMR genes that occur in transposons, plasmids, and integrons that, when mobile, can induce their dissemination [3].
12.2. MINIMIZING THE RISK OF ANTIMICROBIAL RESISTANCE IN ANIMALS AND HUMANS – THE WAY FORWARD

12.2.1. World Health Organization (WHO) and other international body initiatives for the containment of antimicrobial resistance in animal health

- In 1997, the World Health Organisation (WHO) met in Berlin to discuss the medical impact of the use of antimicrobials in food animals (WHO 1997) [1].
- In 2000, the WHO met in Geneva to discuss the global significance of AMR in food animals (WHO 2000) [1].
- In 2003/2004, WHO established the holistic and multi-sectoral approach (One Health) to address the rising threat of AMR. Addressing AMR requires a holistic and multi-sectoral (One Health) approach because antimicrobials used to treat various infectious diseases in animals may be the same or be similar to those used in humans. WHO, the Food and Agriculture Organization (FAO) and the World Organisation for Animal Health (OIE) therefore collaborated to form a tripartite alliance to take collective action to minimize the emergence and spread of AMR [1].
- The first WHO list of CIA was developed in the 1st WHO Expert Meeting on Critically Important Antimicrobials for Human Health held in Canberra, Australia, 2005. During the meeting, participants considered the list of all antimicrobial classes used in human medicine and categorized antimicrobials into three groups of critically important, highly important, and important based on the two criteria developed during the meeting. Table 3 shows the list and classification of antimicrobials that are important in human medicine.
- The Ad hoc Codex Intergovernmental Task Force on Antimicrobial Resistance (TFAMR) was active from 2007–2011 to assess the risks to human health associated with the presence in food and feed including aquaculture and the transmission through food and feed of antimicrobial resistant microorganisms and AMR genes and to develop appropriate risk management advice based on that assessment to reduce such risk [1].
- In 2008, the Advisory Group to the Integrated Surveillance of Antimicrobial Resistance (AGISAR) was established, using the One Health approach [1].
- WHO/Food and Agriculture organisation (FAO)/OIE tripartite alliance’s Global Action Plan (GAP) for AMR was published in 2015. All
member states of the World Health Organisation (WHO) and the Office International des Épizooties (OIE) committed their countries to developing and implementing National Action Plans to control AMR based on the GAP. The GAP strategic objectives are to:

- improve awareness and understanding of AMR through education and training,
- strengthen knowledge and evidence based on surveillance and research,
- reduce the incidence of infection through effective hygiene and infection and prevention control (IPC) measures,
- optimize the use of antimicrobial medicines in human and animal health,
- ensure sustainable investment through research and development [1].

In 2017, the integrated surveillance of AMR in foodborne bacteria using the One Health approach was developed and published with the support of the AGISAR to assist countries and other stakeholders in the establishment and development of programmes of integrated surveillance of AMR in the foodborne bacteria (i.e., bacteria commonly transmitted by food) [5]. There is a schematic representation in Figure 3 of the initiatives undertaken by WHO to address AMR.

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**Figure 3.** Initiatives by the World Health Organization (WHO) for the containment of AMR [6]
Table 3. List and classification of antimicrobials important for human medicine [7]

<table>
<thead>
<tr>
<th>Antimicrobial class</th>
<th>Example of drug(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRITICALLY IMPORTANT ANTIMICROBIALS</strong></td>
<td></td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>gentamicin</td>
</tr>
<tr>
<td>Ansamycins</td>
<td>rifampicin</td>
</tr>
<tr>
<td>Carbapenems and other penems</td>
<td>meropenem</td>
</tr>
<tr>
<td>Cephalosporins (3rd, 4th, and 5th generation)</td>
<td>ceftriaxone, cefepime, cefaroline</td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>vancomycin</td>
</tr>
<tr>
<td>Glycylcyclines</td>
<td>tigecycline</td>
</tr>
<tr>
<td>Lipopeptides</td>
<td>daptomycin</td>
</tr>
<tr>
<td>Macrolides and ketolides</td>
<td>erythromycin, telithromycin</td>
</tr>
<tr>
<td>Monobactams</td>
<td>aztreonam</td>
</tr>
<tr>
<td>Oxazolidinones</td>
<td>linezolid</td>
</tr>
<tr>
<td>Penicillins (natural, aminopenicillins, and antipseudomonal)</td>
<td>ampicillin</td>
</tr>
<tr>
<td>Phosphonic acid derivatives</td>
<td>fosfomycin</td>
</tr>
<tr>
<td>Polymyxins</td>
<td>colistin</td>
</tr>
<tr>
<td>Quinolones</td>
<td>ciprofloxacin</td>
</tr>
<tr>
<td>Drugs used solely to treat tuberculosis or other mycobacterial diseases</td>
<td>isoniazid</td>
</tr>
<tr>
<td><strong>HIGHLY IMPORTANT ANTIMICROBIALS</strong></td>
<td></td>
</tr>
<tr>
<td>Amidinopenicillins</td>
<td>mecillinam</td>
</tr>
<tr>
<td>Amphenicolcs</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>Cephalosporins (1st and 2nd generation) and cephamycins</td>
<td>cefazolin</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>clindamycin</td>
</tr>
<tr>
<td>Penicillins (anti-staphylococcal)</td>
<td>oxacillin</td>
</tr>
<tr>
<td>Pseudomonic acids</td>
<td>mupirocin</td>
</tr>
<tr>
<td>Raminofenazines</td>
<td>clofazimine</td>
</tr>
<tr>
<td>Steroid antibacterials</td>
<td>fusidic acid</td>
</tr>
<tr>
<td>Streptogramins</td>
<td>quinupristin/dalfopristin</td>
</tr>
<tr>
<td>Sulfonamides, dihydrofolate reductase inhibitors and combinations</td>
<td>sulfamethoxazole, trimethoprim</td>
</tr>
<tr>
<td>Sulfones</td>
<td>dapsone</td>
</tr>
</tbody>
</table>

12.2.2 Other International Regulatory Initiatives to minimise the risk of antimicrobial resistance in animal and human health

- In the UK it was recommended by the Swann Committee Report (1969) that in legislation, antibiotics must be clearly designated as either “in feed” (i.e. growth promoters) or “therapeutic”, and that growth-
promoting antibiotics should, by definition, have no therapeutic use in humans or animals. This regulation, however, did not include a provision to withdraw approvals of antimicrobial growth promoters (AGPs) should members of the same class at a later time come into use for humans [1].

- From 1986, Sweden was the first of the Scandinavian countries to take the initiative to ban all antimicrobial growth promoters (AGPs). In 1998 Denmark banned AGP use in pig and poultry production [1].

- As a result of the documentation on the use of antimicrobials for growth promotion in food animals leading to the creation of a major food animal reservoir of bacteria resistant to AGPs and also to medically important last resort antimicrobials, such as vancomycin for example, the European Union then imposed a ban on all AGPs that belonged to classes also used in human medicine. Avoparcin was banned in 1997. The EU then also invoked the “Precautionary Principle” in 1998, which propounds that even if there is not enough scientific evidence on the frequency of the risk of a hazard, but nevertheless the hazard is still seen as a possible risk, then this “Precautionary Principle” may be invoked. On this basis, the use of tylosin, spiramycin, virginiamycin and bacitracin were banned as feed additives in EU countries in 1998. Finally, with effect from January 1st 2006, the last four AGPs used in the EU, monensin sodium, salinomycin, flavophospholipol and avilamycin were banned [1].

### 12.2.3 Consequences of banning of AGPs and decreasing antimicrobial use in livestock production

- In Sweden, interestingly enough, the banning of AGPs did not have a detrimental effect on livestock production. For instance, in the production of slaughter pigs, specialized beef, and turkeys, no negative clinical effects were reported as a consequence of the ban. In piglet production, significant clinical problems emerged that created a demand for antibiotic medicated feed at therapeutic dosages. During the subsequent 4-year period, the use of therapeutic antimicrobials increased, involving up to 75% of the pigs. Thereafter, the use of antimicrobials decreased because of improved management, and was halved in 1993 followed by a gradual further decrease supported by the addition of zinc oxide to the feed. In 1998/1999, only 5% of weaning piglet producing herds used antimicrobial medicated feed and 17% used zinc. The AGP ban has shown that under good production conditions it is possible to reach good and competitive production results for the rearing of rearing of poultry, calves, and pigs without the continuous use of AGPs [1].
A systematic review and meta-analysis was done on the effect of interventions to restrict antibiotics in food-producing animals and its associations between antibiotic resistance in food animals and humans. It was concluded that interventions that restrict antibiotic use in food-producing animals are associated with a reduction in the presence of antibiotic-resistant bacteria in these animals. A smaller body of evidence suggests a similar association in the studied human populations, particularly those with direct exposure to food-producing animals. The implications for the general human population are less clear however, given the low number of studies. These overall findings have directly informed the development of WHO guidelines on the use of antibiotics in food-producing animals [8].

12.2.4 Antimicrobial use and resistance surveillance programmes

With the development of resistance being inevitable, there is a need for the regular monitoring of both use trends as well as to ascertain changes in resistance patterns. The epidemiological data gathered can then be used to determine resistance patterns in a specific area in order to provide information that can be included in future treatment protocols. The goal of antimicrobial monitoring and surveillance should therefore be aimed at providing resistance trends that will ultimately be used to evaluate resistance containment interventions. Surveillance programmes need to be adapted to the resources and facilities specific to the various countries and the following are important factors to consider when setting up a surveillance system for antimicrobial use and patterns of resistance [9]:

- Country based – developing countries versus developed countries.
- Monitoring needs to be continuous and long term in order to give an accurate reflection of trends of antimicrobial use and resistance.
- It is essential that the One Health integrated approach is used to triangulate antimicrobial use and the molecular epidemiology of AMR in the food safety bacteria from humans, the food production continuum from farms to retail meat products and associated sewage and water treatment plants.
- There needs to be national harmonisation of sampling, testing methodologies and validations for antimicrobial patterns of resistance.
- Quality Control must also be implemented when establishing and using these surveillance systems.
- Such systems should be flexible as a surveillance programme is a dynamic system that may change as a result of changed circumstances in the country as well as changed patterns of antimicrobial use and resistance.
Such surveillance programmes should be driven by the government, whether this is the Ministry of Health or Agriculture and it should be made mandatory for the veterinary pharmaceutical companies to provide the relevant sales statistics of antimicrobials reflected in kg of active pharmaceutical ingredient (API), according to the class of antimicrobial.

There are surveillance systems in various countries dealing with volumes of veterinary antimicrobials consumed and AMR trends in animals, food, humans and the environment:

- **Swedish Veterinary Antimicrobial Resistance Monitoring (SVARM)** is a surveillance system for the volumes of veterinary antimicrobials (both food and companion animals) consumed in Sweden as well as AMR trends. This system is part of a holistic approach to limit development of AMR by means of surveillance of AMR and use, control and preventive measures, education, research and training through an agency called the Swedish Strategic Programme for the Rational Use of Antibiotics and Surveillance of Resistance (STRAMA) and is funded by the Swedish government [1].

- **Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP)** was established in Denmark to monitor trends in resistance among bacteria from animals, food and humans, to monitor the consumption of antimicrobial agents and to determine the association between consumption and occurrence of resistance and to model transmission of resistance from animals to humans [1].

- In the United Kingdom, veterinary pharmaceutical companies submit data annually to the Veterinary Medicines Directorate (VMD) on their previous year’s sales of antibiotics authorized for use in animals. The VMD use the data provided to calculate the volume of active antibiotic ingredient within the medicines sold. Sales data are used as an estimate for antibiotic usage. However, as not all antibiotics sold will be used, sales figures are generally an overestimate. The VMD collates data from government laboratories on antibiotic resistance in bacteria found in samples from animals. This is managed through two programmes: EU Harmonised Monitoring, which is carried out as a legal requirement, and a clinical surveillance programme, which relies on voluntary submission of samples by farmers and veterinary surgeons. EU Harmonized Monitoring involves the collection of samples from healthy livestock. Samples are tested for the presence of antibiotic resistant bacteria. The bacteria of interest are those which can potentially transfer between animals and man (zoonotic organisms) [1].

- In Australia, trends in the level and type of antimicrobial use are assessed from import records. These records may include information...
on the indications for which the imported antimicrobials will be used. Importers of antimicrobials (merchants, pharmaceutical companies and private individuals) must hold a permit issued by the Therapeutic Goods Administration (TGA) to import antimicrobials. Since 1992, all importers have had to declare the indication of the antimicrobial that they are importing, whether it is intended for human therapeutic use, veterinary therapeutic use, as a growth promoter, research in the laboratory or for another special purpose. The commonwealth Department of Health and Aged Care, through the TGA is responsible for collecting and reporting these end-use data. The TGA issues permits, collects and maintains this information in an electronic system in a tabulated format. The information is expressed as kg of active ingredient but does not accurately reflect the difference in potencies between agents [1].

- In the United States, surveillance of AMR in food-borne bacteria is undertaken by the National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria [1].

12.2.5 Reducing the need for antimicrobials in animal husbandry

Treatment of infections with antimicrobials is just one facet of animal husbandry and by adopting the following non-antibiotic best practices animal health may be improved while at the same time the use of antimicrobials is decreased and optimized [2]:

- **Improving hygiene practices**
  - Ensure appropriate sanitation and/or disinfection practices.

- **Management**
  - Consistent access to clean water and dietary adjustments.
  - Practice sustainable internal and external parasite control.
  - Introduction of prebiotics, probiotics, enzymes, essential oils etc.
  - Determine the appropriate stocking density to maintain health and welfare.
  - Monitor disease, and maintain accurate and up to date mortality and medication records.
  - Introduce specific pathogen free (SPF) flocks or herds.

- **Biosecurity**
  - Ensure biosecurity of operations by isolation/fencing/visitor control.
  - Optimize vaccination programmes.
Veterinary antimicrobial stewardship

- Continuously monitor and measure biosecurity and be proactive in improving biosecurity based on these assessments.

- **Accountability with use of antimicrobials**
  - Ensure that there is a responsible, qualified and knowledgeable person in charge of the use of antimicrobials, particularly in intensive operations, such as a veterinarian or other trained person.

### 12.2.6 Alternatives to antibiotics

#### 12.2.6.1 Background

The golden age of the discovery, development and commercialization of new classes of antibiotics and novel antibiotic technologies was during the 1960s and 1970s. However since then research and development of new antibiotics has declined. The reasons for this decreasing interest by the pharmaceutical industry are multifactorial. Bringing a new drug to market requires an average investment of US$800 million and 10 years or longer. As well, pharmaceutical companies have to support the relatively large research costs of medications that do not make it to market. Furthermore, the risks of post-approval adverse events must also be taken into consideration. Another factor that may play a role in the reduced interest in antibacterial development is the current focus on medications for the treatment of chronic diseases. Unlike medications used to treat chronic diseases, most antibiotic treatments are given for 5 to 14 days and then discontinued. Anti-infectives are intended to quickly eliminate the need for their use. In addition, novel breakthrough antimicrobials often become the agents of last resort, as clinicians and policy makers tend to hold them in reserve, hoping to slow the inevitable emergence of resistance. Also, antibiotics work very well and quickly, and therefore they produce a low return on investment for manufacturers [10].

There have been recent developments in novel technologies that could potentially lead to alternatives to antibiotic use. The feasibility of such alternatives needs to be analysed in depth to ascertain if any of these could possibly substitute veterinary antibiotics in the future. Some of these alternatives include antibacterial vaccines, immunomodulatory agents, bacteriophages and their lysins, antimicrobial peptides (AMPs), pro-, pre-, and synbiotics, plant extracts, inhibitors for bacterial quorum sensing (QS), biofilm inhibitors and plant extracts [11].

Ideal alternatives to antibiotics should: (i) have non-toxic or no side effects on animals, (ii) be easy to eliminate from the body or result in short term residues, (iii) not induce bacterial resistance, (iv) be stable in the feed and animal gastrointestinal tract, (v) be easily decomposed and not affect the environment, (vi) not affect palatability, (vii) not destroy the normal intestinal flora of animals, (viii) kill or inhibit the growth of pathogenic bacteria, (ix) enhance the body’s resistance to the disease, (x) improve feed efficiency and
promote animal growth, and (xi) have good compatibility. In fact, there are no alternatives to antibiotic that currently meet all the above mentioned requirements [11].

12.2.6.2 Antibacterial vaccines
There is still a considerable gap between antibiotic alternatives and antibiotics concerning the effectiveness of disease prevention and growth promotion. Antibacterial vaccines are generally used for the prevention of bacterial infections, and currently only a small number of bacterial infective diseases can be controlled by vaccines. The development of a vaccine that is both practical and inexpensive so that it can be affordable for use in poor countries is still a key problem. The most important challenge for mass immunization with poultry vaccines is the cost of vaccine as well as the feasibility. While vaccines may lessen our reliance on the use of antibiotics, they are complementary rather than a replacement [11].

12.2.6.3 Immunomodulators
Immunomodulators, mainly immunostimulants, are able to non-specifically enhance the innate immune function and to improve the host’s resistance to disease. The use of immunotherapy in infectious diseases may resulting in modulating the immune response to a microbe (e.g., by using cytokines and cytokine inhibitors), modifying a specific antigen-based response (e.g., using interferons) and minimizing end-organ damage using non-specific anti-inflammatory agents (e.g., steroids). Immunomodulators and feed enzymes mainly preserve the health of animals, but do not directly kill or inhibit bacteria [11].

12.2.6.4 Bacteriophages
Bacteriophages are viruses that are parasitic on bacteria. Bacteriophages are currently only used in food, and the safety is still questionable [11].

12.2.6.5 Probiotics
Probiotics have been defined by the WHO as “microorganisms which, administered live and in adequate amounts, confer a benefit to the health of the host.” Probiotics can kill and prevent pathogenic microorganisms by producing antimicrobial compounds such as bacteriocins and organic acids, improve gastrointestinal microbial environment by adherence to intestinal mucosa thereby preventing attachment of pathogens and competing with pathogens for nutrients, stimulate the intestinal immune responses and improve the digestion and absorption of nutrients [11]. There is a diagrammatic representation of the action of probiotics in the animal intestinal tract in Figure 4.
12.2.6.6 Plant extracts

Plant materials are used widely in traditional systems of medicine. Plant extracts, also known as phytobiotics, have been exploited in animal nutrition, particularly for their antimicrobial, anti-inflammatory, anti-oxidative, and anti-parasitic activities. However, the composition of plant extracts and probiotics is complex and the quality in terms of stability is poor, resulting in varying effects and safety risks [11].

![Figure 4. Mechanism of action of probiotics within the intestinal tract of animals [12]](image)

12.2.6.7 Prebiotics

Prebiotics are non-digestible (by the host) food ingredients that may have a beneficial effect through their selective metabolism in the intestinal tract. Prebiotics include oligosaccharides, polysaccharides, natural plant extracts, protein hydrolysates, polyols, etc. Prebiotics are stable compounds with no residues, no induced resistance, and may be supplied from a wide variety of sources. However, many prebiotic products are not authorized currently in the EU as feed additives under the commission regulation (EC) 1831/2003. This is due to certain drawbacks to these products. Firstly, prebiotics themselves cannot inhibit and kill pathogens, thus they cannot prevent or treat bacterial infections as antibiotics do. Secondly, feeding a large quantity of prebiotics may cause bloating, diarrhea, and other adverse reactions in livestock due to the fermentation in the gastrointestinal tract [11].
12.2.6.8 Synbiotics

Synbiotics are the joint preparations of probiotics and prebiotics, and therefore have their dual role. There are some reports on the beneficial effects of synbiotics on the physiological and production indices of piglets such as the enhancement of production performance in piglets and the improvement of average daily gain. However, the data from these reports on the beneficial effects of synbiotics in swine production are still limited [11].

12.2.6.9 Quorum sensing inhibitors

Bacterial pathogenicity is, in part, under the regulation and control of the QS system. A QS system consists of self-induced signaling molecules (auto-inducers [AIs]), receptors, and downstream regulatory proteins. Inhibitors targeting the QS system can block its functions and thereby inhibit the bacterial pathogenic effects controlled by the QS system. Inhibitors targeting QS and pathogenicity of bacteria are still being researched with no approved products, and most QSIs are toxic to eukaryotic cells. Biofilms are structured consortiums of bacteria embedded in a self-produced polymer matrix consisting of polysaccharide, protein and DNA. Biofilm-forming bacteria may cause chronic infections because they show increased tolerance to antibiotics and disinfectants as well as resisting phagocytosis and other defense mechanisms of the body. Biofilm inhibitors show good results only when used in combination with antibiotics. AMPs include products such as gramicidin, polymyxin, bacitracin, and sugar-peptide. Although AMPs can treat bacterial infections, the high cost and narrow antibacterial spectrum restrict their wide use, and they can still induce bacterial resistance [11].

Antibiotics can directly inhibit or kill bacteria with better antibacterial effect than all the antibiotic alternatives mentioned here. Moreover, antibiotics are formulated from relatively pure active ingredients and the quality is ensured by good manufacturing practice. So far, no antibiotic alternative has satisfactorily replaced the clinical efficacy of appropriate antibiotics used in bacterial infections [11].

12.2.7 Other actions to consider and take

The question to be asked is whether any antimicrobial use can select for resistance i.e. sub-therapeutic levels over an extended period in the feed for growth promotion versus high doses? The World Veterinary Association (WVA) has recommended ‘use as long as needed but for the shortest duration possible [2].

Other actions to take for an integrated approach to veterinary antimicrobial stewardship are:
• Prudent use guidelines to be adopted or adapted from international bodies such as the OIE that are applicable and practical to all the various animal species in the various countries [2].

• The Veterinary International Conference of Harmonisation (VICH) is a trilateral (EU-Japan-USA) programme aimed at harmonising technical requirements for veterinary product registration. The VICH GL27 is the Guidance on Pre-Approval Information for Registration of New Veterinary Medicinal Products for Food Producing Animals with Respect To AMR is one of the VICH guidelines. It is recommended that pre-registration requirements for antimicrobials be in accordance with this VICH guideline. Furthermore, the Regulatory Authorities (RAs) also play a pivotal role in AMR by determining local registration requirements, availability of antimicrobials on the market as well as access to antimicrobials (for example, control by veterinarians). RAs may determine that local Minimum Inhibitory Concentration (MIC) data be collated for a representative sample of the bacteria for which claims are made, withdrawal periods to be set in food animals, flagging and evaluation of any potential antimicrobials for resistance that may impact on human health, and pharmacokinetic/pharmacodynamic (PK/PD) relationships of antimicrobials to optimize the use of concentration dependent bactericidal versus time dependent bactericidal antimicrobials in order to maximize safety and efficacy but minimize the risk of selection for resistant bacterial populations [13].

• Some countries have a dual registration system of over the counter (OTC) antimicrobials versus veterinary prescription only which leads to inconsistencies in registration requirements. Such systems need to be evaluated with the view to having one regulatory agency to control antimicrobials or harmonised registration requirements for both OTC and scheduled veterinary antimicrobials [13].

• Do not use critically important and highly important antimicrobials to human medicine, except for treatment and when there is no alternative [14].

• Ensure that no compounded medicines are used in food animals or extra label use of medicines, except as a last line resort. If antimicrobials are compounded as a last resort for food animals, then Good Manufacturing Practice (GMP) or Good Pharmacy Practice (GPP) need to be implemented during the production to ensure the highest quality medicine possible. A suitable withdrawal period also needs to be established by the veterinarian to ensure that the consumer is not exposed to any antimicrobial residues that may lead to AMR [14].

• The Pharmaceutical Inspection Co-operation Scheme (PIC/S) leads the international development, implementation and maintenance of harmonised GMP standards and quality systems of Inspectorates in the
field of medicinal products. It is important for countries to be part of such systems in order to standardise the quality of medicines globally [15].

- There need to be incentives for the research and development of novel antibiotics, such as research grants [10].
- Use pharmacovigilance as a tool to also establish AMR trends as a lack of efficacy of an antimicrobial is also classified as an adverse drug reaction (ADR).
- The Regulatory Authorities need to be very vigilant on preventing counterfeit medications from reaching the market as such medicines may not have the correct API, or the incorrect quantity of API or may be of sub-standard quality amongst other issues that may contribute to the AMR problem.
- The undergraduate and postgraduate curriculum of all animal science, medical science and veterinary students needs to introduce more information on the importance of rational antimicrobial use to minimize the development of AMR and the interrelationships between AMR in humans, animals and food products and the environment [1,13].
- The efficacy of traditional antibiotics can still be improved. Some “old” antibiotics can find new bacterial targets and be used against some multi-drug resistant (MDR) bacteria. It has been demonstrated that in many cases, there are non-carbapenem alternatives for the treatment of extended-spectrum-β-lactamase-producing E. coli (ESBL-Ec) infections. Novel formulations can also allow targeted drug delivery via nanoparticles to reinforce the antimicrobial effect of these antibiotics. Furthermore, in empirical therapy, use of broad-spectrum bactericidal agents that will eradicate the presumed infective microorganism(s), which potentially could be MDR, should be selected. Once an infection is under control and the culture and susceptibility results are reported, it is important to then switch to the most suitable narrow-spectrum antibiotic thereby decreasing the potential of adverse drug effects and the risk of development of antibiotic-induced resistance [11].
12.3. CONCLUSION

We must not forget that “prevention is better than cure”. However, for many developing countries, because of the poor farming environment and the high incidence of disease, antibiotics are still an effective tool in the prevention and control of animal diseases. It was proven that the ban of growth promoters in EU resulted in a requirement for the improvement of the farm hygiene. After the amount of “old” antibiotics used in feed was reduced due to the ban, the prevalence of bacterial infections in the target animals was shown to increase if there was no fundamental improvement of hygiene and animal husbandry in the production environment. This may lead to increased therapeutic use of antibiotics and result in some unintended consequences that would be cause for new public health concerns. The benefit versus risk should be assessed before implementing such policies as a result of political/social pressure, as bacteria may not necessarily “listen” to the policy-makers. Thus, the decision concerning the use of in-feed antibiotics should be made based on scientific approaches. The ban of antibiotics as growth promoters cannot be implemented in every country of the world [11].

Again, the integrated One Health approach to antimicrobial stewardship and resistance needs to be emphasized. All stakeholders, from the various governmental agencies, veterinary pharmaceutical industry, veterinarians, para-veterinarians, agricultural sector, food processors, abattoirs, retail shops, animal scientists, medical profession, companion animal owners and the public need to work together as a cohesive whole, in order to resolve this crisis [13]. Antibiotics must be used responsibly and there must be continuous development of alternatives to antibiotics to ensure the long-term sustainability of the existing antibiotics. Antimicrobial use with respect to target animals, duration of the treatment, withdrawal period, prudent use as well as regulations/policies must be strictly defined. At the same time, the supervision and enforcement of such laws must be strengthened in order to control antibiotic resistance. It is essential that antimicrobial use and resistance surveillance programmes are implemented that are applicable, sustainable and practical to the socioeconomic development and agricultural policies of the various countries. Furthermore, we must improve the management of animal nutrition and production hygiene, since recent European developments showed a distinctly more positive outcome from the ban of antibiotic growth promoters than was anticipated due to the improvement of animal welfare.

It is absolutely essential that the awareness of AMR is now translated into the action of veterinary antimicrobial stewardship in order to preserve the efficacy of our antimicrobials for our future generations to also enjoy the good healthcare that they have a right to receive!
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INHIBITION OF BACTERIAL STRESS RESPONSES – STATE OF THE ART

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13.1. INTRODUCTION

Microorganisms are amazing, not only for the ability of different species to survive and grow in almost all places in this world, but also in the flexibility of species to respond and adapt to multiple adverse environmental stressors, such as extreme temperature/pH/osmotic pressure, nutrient starvation, and presence of toxins like antimicrobial agents. Adaptation via environmental sensing involves a complex transcriptional regulatory network that enables microorganisms to systematically alter expression of a wide variety of genes, enhance frequency of mutations facilitating the appearance of beneficial ones, and promote microbial collaboration to ensure their survival and growth. In this chapter, we will first provide an overview of up-to-date understanding of bacterial stress responses with focus on antimicrobial stress and, second, summarise efforts to inhibit antimicrobial stress response as a potential way to fight antimicrobial resistance (AMR).

13.2. BACTERIAL STRESS RESPONSES AND THEIR IMPACT ON ANTIMICROBIAL RESISTANCE

13.2.1. Antimicrobial stress response

AMR, the ability of microbes to resist the effect of drugs working against them, is now a serious global problem threatening public health regardless of borders. Without effective antimicrobials, the success of major surgery, cancer therapy, and control of community-acquired infections would be strongly reduced. However, it has been observed that sooner or later after the introduction of new antibiotics, resistance in bacterial isolates will arise. One factor that contributes significantly to this issue is the activation of the stress response in the presence of antimicrobials. For example, with Pseudomonas aeruginosa infections, aminoglycosides such as tobramycin or amikacin are frequently used. Although the most common mechanism of aminoglycoside resistance involves aminoglycoside-modifying enzymes encoded by transmissible genes that are acquired by horizontal gene transfer [1], the predominant resistance mechanisms occurring in persistent bacterial infections in response to antibiotic treatment, such as in case of cystic fibrosis, is impermeability-type pan-aminoglycoside resistance. This involves the MexXY-OprM multidrug efflux system and lipopolysaccharide (LPS) modification [2], which is regulated by the PhoPQ two-component system (TCS), an important sensor in multiple stress responses [3]. PhoPQ TCS, consisting of histidine kinase, PhoQ, and the response regulator, PhoP, are also
known to respond to cationic antimicrobial peptides such as polymyxin B, pH, and divalent ion starvation such as Ca$^{2+}$ and Mg$^{2+}$. Genes downstream of PhoPQ are responsible for membrane strengthening via phospholipid and LPS modification. PhoP also activates PmrAB TCS, which in turn leads to lipid A modification, resulting in increased hydrophobicity and decreased permeability [4]. Recently, it was reported that PhoPQ also contributes to resistance of pathogens to reactive nitrogen species (RNS) [5].

One TCS that is also triggered by antimicrobials such as aminoglycosides is CpxRA, an envelope stress TCS. CpxRA comprises a sensor histidine kinase (CpxA) and a cytoplasmic response regulator (CpxR) [6]. It promotes reduced susceptibility to aminoglycosides, beta-lactams, novobiocin, and cationic antimicrobial peptides partly due to CpxRA-dependent upregulation of multidrug efflux, peptidoglycan amidase genes, and porins [7-9].

Another TCS response regulator, AmgrS, which is activated under aminoglycoside exposure, apparently regulates envelope/membrane stress response (ESR). This regulator has been linked to the control of numerous membrane transporters and protease genes whose products can restore aminoglycoside-generated mistranslated polypeptides resulting in aminoglycoside resistance [10]. It is, thus, described as a determinant of intrinsic aminoglycoside resistance [11].

Although TCS is more prevalent in Gram-negative bacteria, TCSs have been increasingly reported in Gram-positive bacteria, among which many are also activated under antimicrobial exposure. For example, CroRS, which is induced under the presence of beta-lactams and other cell wall synthesis inhibitors such as bacitracin, fosfomycin, and vancomycin, is responsible for the production of modified penicillin-binding proteins, thus resulting in beta-lactam resistance. GrasRS (so called Aps) responding to cationic antimicrobial peptides contributes to resistance to polymyxin B, daptomycin, and vancomycin via membrane modification [12-14]. Two other regulators known to be activated in response to membrane-disrupting agents are LiaRS and NsaRS. These regulate a number of genes, particularly those involved in cell-wall synthesis, thus contributing to AMR. Data have shown that mutation of these regulators results in markedly reduced ability to develop AMR [15,16].
Envelope stress response (ESR) or membrane stress response is a response to envelop/membrane damage. It is one of the most important stress responses in both Gram-negative and Gram-positive bacteria regarding the essential function of the bacterial envelop as a sensor and protective interface and its vulnerable structure as the target of numerous harmful factors including antibiotics. ESR is controlled mainly by sigma E and the Cpx TCS.

One apparent effect of antimicrobials, particularly the bactericidal agents such as ampicillin, gentamicin, and norfloxacin, is to facilitate the production of reactive oxygen species (ROS) and oxidative stress inside exposed cells, which can kill the bacteria (Dwyer et al. 2014). However, the oxidative stress response can also promote AMR. It can do this indirectly by promoting ROS-induced mutations that result in development of AMR. It could also promote resistance via activation of regulators such as AsrR, which regulates 181 genes including those involved in antibiotic and antimicrobial peptide resistance [17,18]. It can do this indirectly by promoting ROS-induced mutations that result in development of AMR. It could also promote resistance via activation of regulators such as AsrR, which regulates 181 genes including those involved in antibiotic and antimicrobial peptide resistance [17,18]. It could also promote resistance via activation of regulators such as AsrR, which regulates 181 genes including those involved in antibiotic and antimicrobial peptide resistance [17,18].

Besides the various antimicrobial-induced responses that are responsible for AMR development mentioned above, genomic/transcriptomic investigations have also shown that antibiotics are able to induce expression of various gene classes associated with the SOS response [29-31], heat shock [32-34], acid stress response [35], and multiple other genes involving in metabolism and virulence [36]. Ciprofloxacin, for instance, induced genes for the SOS response, including dnaT, rimM, and recN, genes for cytolysis, genes for biofilm formation, genes of the tricarboxylic acid (TCA) cycle, and multiple other genes that contribute to the resistance to ciprofloxacin and other antibiotics [29-31]. Sub-inhibitory concentrations of streptomycin and beta-lactam antibiotics were shown to successfully induce DnaK and GroEL, two principal heat shock proteins that enable bacteria to tolerate high temperatures [33]. In response to tobramycin, the expression of a number of heat shock genes are induced, particularly in anaerobic conditions under processes requiring the presence of the heat shock sigma factor RpoH [34]. In this study, marked alteration in the expression of multiple gene groups under the presence of tobramycin was shown [34]. Recent genome-wide transcriptional analysis of the response in E. coli to antibiotics of diverse mechanisms of action, trimethoprim (folate synthesis inhibitor), tetracycline (30S inhibitor), nitrofurantoin (a RNS), and chloramphenicol (50S inhibitor), found that they induce diverse gene expression changes with variable levels [35]. While 20% of the 1,000 tested...
library promoters were up- or down-regulated by more than 2-fold for trimethoprim and tetracycline, only 5% responded to chroramphenicol. The early oxidative response was observed to be triggered by tetracycline and nitrofuratonin while the delayed SOS response was induced by trimetroprime and nitrofuratonin. Furthermore, a clear and strong acid stress response was induced by trimethoprim, which then proved to protect the bacteria from subsequent HCl challenge [35].

Overall, it is undeniable that antibiotics can trigger bacterial responses that can lead to alteration of cell physiology, affecting not only its ability to resist antibiotics but also its virulence and fitness upon subsequent environmental challenge. This kind of cross-protection certainly can complicate bacterial infection treatment not only in terms of resistance to antibiotic therapy but also in the way that pathogens develop resistance to the host immune system and increase their virulence.

13.2.2. Other stress responses

As antimicrobials are only one of various environmental challenges exerted upon bacteria and, generally, all responsive adaptations of bacteria are in a complex unified network, response to stress stimuli other than antimicrobials will have an impact on antimicrobial susceptibility.

One common environmental challenge to bacteria is nutrient starvation, which is widely accepted as a promoting factor, causing bacteria to become highly tolerant to antimicrobials [37]. For instance, it has been observed that amino acid starvation can result in resistance to ampicillin and ofloxacin; while depletion of glucose leads to resistance to protein synthesis inhibitors such as gentamicin [38]. It was also observed in many bacterial pathogens, such as *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and biofilm producers, that oxygen limitation is an important factor inducing AMR [39-41], and introducing oxygen to anoxic ones can increase their susceptibility to antimicrobials [42]. In a number of bacteria, low levels of divalent cations, particularly Mg^{2+}, can significantly reduce their antimicrobial susceptibility [43]. This is explained by the fact that divalent cations have a central role in stabilising bacterial membranes.

Mechanisms leading to starvation-induced AMR are thought to include the inactivity of antibiotic targets caused by starvation-induced growth arrest and, at the same time, an active response to starvation, starvation stress response, or stringent response. It is known that deficiency in carbon, amino acids, and iron activates relA and spoT, which results in production of the alarmone, (p)ppGp. This signal, in turn, leads to altered expression of a wide range of genes, including those responsible for AMR. It has been shown that inactivating the starvation stress response, via disrupting relA or spoT for example, can markedly enhance the efficacy of antibiotics [44].
Sub-lethal stresses of pH, NaCl, temperature, or metals also significantly alter antibiotic resistance. A study on food-related pathogens including *Escherichia coli*, *Salmonella enterica serovar Typhimurium*, and *Staphylococcus aureus* showed that, while reduced pH (< 5.0) and increased salt (> 4.5 % w/v) increased AMR, sub-lethal high temperature (45 °C) decreased it [45]. The observed decrease was probably due to the fact that, in this study, bacteria were incubated at high temperatures (45 °C) for a longer time, indicated as “until no increase was observed in optical density (OD) readings”, than in other studies on heat shock (cultured at optimal temperature followed by 30 min at 40–45 °C). Temperature stress in the form of heat shock generally induces AMR. A study in *E. coli* showed that expression of ClpL, a major heat shock protein induced PBP2x expression, resulted in reduced penicillin susceptibility [46]. A heat shock of 45 °C for 30 min on *A. baumannii* also increased survival of the bacteria under streptomycin exposure, which seemed linked with DnaK and GroEL, two important heat-shock proteins [33].

Although some metals, like Cu and Zn, are essential for microbial growth, at high concentrations they can be toxic or exert selective pressures on bacteria, which could produce multidrug resistance profiles including resistance against carbapenems and third generation cephalosporins [47]. Interestingly, the applied sub-lethal stress was shown to induce stable increases in AMR.

### 13.2.3. Concluding remarks

Antimicrobial and non-antimicrobial stress responses have a strong impact on the development of AMR, which can have serious effects on treatment due to failure of antibiotic therapy. A better understanding of stress response networks and the specific genes involved would be very important in order to target them therapeutically.

In addition to the target proteins mentioned above, it would be a big mistake not to mention essential controllers for gene expression, which are RNA polymerase and corresponding sigma factors. The association/disassociation of these two proteins determines the transcriptional level of a gene. Under specific conditions, a certain sigma factor can integrate with RNA polymerase to form an active holoenzyme that is able to recognise a certain promoter site and transcribe subsequent genes. Most bacteria have a number of sigma factors responding to particular stresses and directing RNA polymerase to transcribe required target genes. Apart from primary or housekeeping sigma factors, which control bacterial growth and metabolism, the alternative ones, such as sigma B (SigB), sigma E (RpoE), sigma S (RpoS), sigma 32 (RpoH), AlgU/T, CarQ, etc., regulate specific physiological processes including general stress responses. Among these alternative sigma factors, the most widespread and important sigma factor group responsible for responding to various dynamic environmental signals is the extracytoplasmic function (ECF) group. Most ECF sigma factors, like sigma E, are transcribed with their cognate
negative regulators, which bind to and inhibit them and only under environmental stimuli are ECF sigmas released and bind to RNA polymerase to activate transcription of specific target genes [48]. Today, ECF sigma factors are considered regulons for stress survival, virulence, and antibiotic resistance in many pathogens. That a single sigma factor can control a hundred target genes opens the possibility to target sigma factors to regulate systemic cellular responses, particularly in coping with AMR and virulence developed under antibiotic or environmental stress stimuli.

Over the past years, there has been increasing evidence showing that small, non-coding RNAs or regulatory RNAs are key players in stress responses, regulating a variety of stress response pathways via transcriptional and post transcriptional control. Regulatory RNAs comprise two major classes, RNA attenuators and small RNAs (sRNAs). Attenuators are transcribed as part of the mRNA they regulate and act via attenuating transcription or translation due to their alteration in secondary structure. sRNAs are expressed independently from their targets and include two types, cis- encoded or antisense sRNAs, which are transcribed from the complementary strand of the target gene, thus fully complementary to their target and trans-encoded sRNAs, which are only partially complementary to their distantly encoded target and often require proteins such as CsrA, Hfq, or ProQ for their activity and function [49]. Hundreds of sRNAs have been found in most bacteria, among those many have been proven to have an essential role in regulating the stress response pathway. RyhB in *E. coli*, for example, is induced by the transcription factor Fur under iron depletion and its overexpression results in expression change of more than 50 genes involved in iron storage, iron-sulfur biogenesis, iron-containing proteins, respiration, and siderophore biosynthesis. This effect is mediated by the ability of RyhB to repress target mRNAs of non-essential iron-using proteins, thus freeing irons for essential proteins that rely on iron for their activity [50]. RyhB together with other sRNAs like MicA and RybB contribute to the envelope stress response. These sRNAs, induced by the principal regulator of the envelope stress response sigE, inhibit translation of target mRNAs encoding OMPs and lipoproteins. This kind of regulatory principle is applied for most known sRNAs, including MicF in oxidative, osmolar, and antibiotic stress; OxyS in oxidative stress; SgrS in sugar-phosphate stress; DsrA and RprA in acid stress; TisB in SOS response/DNA damage stress; and multiple sRNAs involved in starvation stress [49,50]. It is not surprising that data recently showed that riboregulation exerts a strong impact on antimicrobial susceptibility with its participation in the complex regulatory network controlling drug influx/efflux and drug target modification and metabolism. The pivotal role of regulatory RNAs in antimicrobial susceptibility makes it possible to improve antibiotic usage via combining inhibitors of regulatory RNAs and/or their protein cognate partners, thus silencing corresponding genes involved in AMR. The genes and regulatory
factors responsible in bacterial stress responses are summarised in Table 1 and present in a networking in Figure 1 (Figure 1, Table 1).

Figure 1. Bacterial stress response network. Genes responding to antimicrobial stress are highlighted.
Table 1. Genes involved in bacterial stress response. Genes in human bacterial pathogens and of importance in antimicrobial stress are listed.

<table>
<thead>
<tr>
<th>Stress</th>
<th>Regulators</th>
<th>Resistance mechanisms</th>
<th>Affected antimicrobials</th>
<th>Organisms</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimicrobials</td>
<td>AdeRS</td>
<td>AdeABC efflux</td>
<td>Tigecyclin, carbapenems, others</td>
<td><em>Acinetobacter baumannii</em></td>
<td>[51]</td>
</tr>
<tr>
<td>CpxRA</td>
<td>AcrBD, eefB efflux</td>
<td>Beta-lactam, chloramphenicol</td>
<td><em>K. pneumoniae</em>, <em>E. coli</em></td>
<td>[9] [52]</td>
<td></td>
</tr>
<tr>
<td>LexA/RecA</td>
<td>QnrB, STX, Intl</td>
<td>Multiple drugs</td>
<td><em>Enterobacteriaceae</em>, Vibrio sp.</td>
<td>[53]</td>
<td></td>
</tr>
<tr>
<td>PhoPQ/PmrAB</td>
<td>Surface remodeling, lipid A modification, biofilm, proteolytic degradation</td>
<td>Colistin, Polymyxin B</td>
<td><em>Acinetobacter baumanii</em>, <em>K. pneumoniae</em>, <em>P. aeruginosa</em>, <em>S. enterica</em>, <em>S. flexneri</em></td>
<td>[54] [55] [53] [56]</td>
<td></td>
</tr>
<tr>
<td>PhoBR</td>
<td>Porin PhoE</td>
<td>Carbapenems</td>
<td><em>K. pneumoniae</em></td>
<td>[57]</td>
<td></td>
</tr>
<tr>
<td>VraSR</td>
<td>Cell wall biosynthesis change: PrsA,FmtA, glycosyltransferase, TcaA</td>
<td>Beta-lactams</td>
<td>Glycopeptides</td>
<td><em>Bacitracin</em>, <em>S. aureus</em></td>
<td>[58] [59]</td>
</tr>
<tr>
<td>Nutrition starvation (iron, oxygen, etc.)</td>
<td>SOS/LexA</td>
<td>Stress-induced, mutagenesis, persisters</td>
<td>Fluoroquinolone</td>
<td><em>E. coli</em>, <em>M. tuberculosis</em>, <em>P. aeruginosa</em></td>
<td>[53,60]</td>
</tr>
<tr>
<td>RpoS, RpoE</td>
<td></td>
<td>Multiple drugs</td>
<td><em>E. coli</em>, <em>M. tuberculosis</em>, <em>P. aeruginosa</em></td>
<td>[61] [62]</td>
<td></td>
</tr>
<tr>
<td>RelA, SpoT ((p)ppGp)</td>
<td></td>
<td>Stringent response-mediated amelioration of antimicrobial-dependent oxidative stress</td>
<td>Multiple drugs</td>
<td><em>P. aeruginosa</em>, <em>E. coli</em></td>
<td>[53] [63]</td>
</tr>
<tr>
<td>Stress</td>
<td>Regulators</td>
<td>Resistance mechanisms</td>
<td>Affected antimicrobials</td>
<td>Organisms</td>
<td>Refs</td>
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</tr>
<tr>
<td>Oxidative/ nitrosative</td>
<td>SoxRS</td>
<td>AcrAB-TolC, WaaYZ</td>
<td>Multiple drugs</td>
<td>E. coli, S. enterica</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[64]</td>
</tr>
<tr>
<td>PA5471</td>
<td>MexXY-OprM</td>
<td>Multiple drugs</td>
<td>P. aeruginosa</td>
<td>[53]</td>
<td>[65]</td>
</tr>
<tr>
<td>MexR</td>
<td>MexAB-OprM</td>
<td>Multiple drugs</td>
<td>P. aeruginosa</td>
<td>[53]</td>
<td>[66]</td>
</tr>
<tr>
<td>MgrA/ SarZ</td>
<td>NorA, NorB, Tet38</td>
<td></td>
<td>beta-lactams</td>
<td>S. aureus</td>
<td>[67]</td>
</tr>
<tr>
<td>MexT</td>
<td>MexEF-OprN</td>
<td>Multiple drugs</td>
<td>P. aeruginosa</td>
<td>[53]</td>
<td>[68]</td>
</tr>
<tr>
<td>pH (Acidic, Alkaline)</td>
<td>SigB ($\sigma^B$)</td>
<td>RsbK-RsbY-RsbV</td>
<td>beta-lactams, glycopeptides</td>
<td>S. aureus</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[69]</td>
</tr>
<tr>
<td>SigE ($\sigma^E$)</td>
<td>YaeL-RseA</td>
<td>Kanamycin, polymyxin B</td>
<td>E. coli, S. enterica</td>
<td></td>
<td>[70]</td>
</tr>
<tr>
<td>Temperature (heat, cold)</td>
<td>RpoH, AsrA</td>
<td>Drug-induced aberrant polypeptide turnover</td>
<td>beta-lactams</td>
<td>S. aureus</td>
<td>[53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. aeruginosa</td>
<td>[72]</td>
</tr>
<tr>
<td>ClpL, PBP2X</td>
<td>PBP2X</td>
<td>ClpL, PBP2X-mediated thickening of cell wall</td>
<td>Penicillin, beta-lactams</td>
<td>S. pneumoniae</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[46]</td>
</tr>
</tbody>
</table>
13.3. INHIBITION OF THE ANTIMICROBIAL STRESS RESPONSE

It is evident that AMR development is unpreventable once antimicrobials are used. In combating AMR, inhibitors of novel targets other than the “established targets” have been sought with little success [74].

Attempts to combat AMR by inhibiting the antimicrobial stress response while using existing antibiotics in a combination therapeutic approach have only recently gained attention. Some progress has been made and this is promising for future developments in this area [22].

Data obtained so far have clearly shown that knock-down or knock-out of important regulators of stress responses significantly enhances activity of existing antimicrobials and reduces the rate of AMR development. For example, blocking the stress regulator AmgRS enhanced aminoglycoside activity in both planktonic and antibiotic tolerant bacteria [22]. Knocking out multiple genes in *E. coli*, particularly those involved in transcriptional regulation (*deoT, dksA*) or SOS genes (*recA, recN*), also increased sensitivity to different classes of antibiotics [75]. In *Staphylococcus aureus*, inactivation of VraS TCS, an envelope stress regulon, caused a marked reduction in resistance to beta-lactam antibiotics and vancomycins [58,59]. In addition, inhibition of the SOS response via the RecA/LexA axis rendered *S. aureus* unable to evolve antibiotic resistance in response to UV damage [29].

In *Acinetobacter baumanii*, the lack of AdeRS TCS resulted in significantly decreased expression of RND efflux genes, including *adeABC* which can result in increased sensitivity to a wide range of antibiotics that they control such as aminoglycosides, beta-lactams, tetracyclines, macrolides, and chloramphenicol [51]. In *Pseudomonas aeruginosa*, mutant strains deficient in *rpoS, relA* and *spoT*, or *anr*, a gene encoding an important mediator of hypoxia stress, had markedly increased susceptibility to ciprofloxacin [31]. Interestingly, recent data also showed that inhibition of the SOS response via disruption of the RecA/LexA axis in *E. coli* with different established quinolone resistant determinants, such as chromosomal gyrA/parC mutations, marR deletion, and plasmid-mediated quinolone resistance (PMQR) qnrS, can reverse the resistance phenotype and has a fascinating effect in an *in vivo* murine model with decreased virulence and increased antibiotic susceptibility [76]. Very recently, attempts at interference with sRNA regulatory pathways showed promising results in increasing antimicrobial susceptibility. For example, the absence of MicF increased susceptibility to cephalosporin and norfloxacin; of GcvB, sensitised bacteria to D-cycloserine; of RyhB, reduced resistance to colistin; and of MgrR/SroC, affected polymyxin B resistance [49]. Compounds are known to interfere with bacterial stress responses are summarized in Table 2 and discussed in details in the followed sections.
Table 2. Compounds with potential to inhibit the bacterial stress response. Compounds having inhibitory activity on the antimicrobial stress response are highlighted.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Pathways</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ions</td>
<td></td>
<td>[80]</td>
</tr>
<tr>
<td>Zinc</td>
<td>SOS response (LexA-RecA)</td>
<td></td>
</tr>
<tr>
<td>Small</td>
<td>VraSR</td>
<td>[78]</td>
</tr>
<tr>
<td>molecules</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-aminoimidazole derivatives, C1, D1, Curcumin</td>
<td>SOS response (LexA-RecA)</td>
<td>[87]</td>
</tr>
<tr>
<td>Suramin/germanin (polysulphonated naphthyl urea), RWJ-49815 (ethyl guanidine derivative)</td>
<td>SOS response (LexA-RecA)</td>
<td>[84,85]</td>
</tr>
<tr>
<td>Polyamine</td>
<td>Histidine kinases of TCSs</td>
<td>[77]</td>
</tr>
<tr>
<td>Cadaverine, Putrescine, Spermidine, Spermine</td>
<td>Oxidative response (oxyR- katG)</td>
<td>[88]</td>
</tr>
<tr>
<td>Peptides</td>
<td>4E1 (18-aa peptide), 1018, DJK-5</td>
<td>SOS response (LexA-RecA); Stringent response-biofilm ((p)ppGpp)</td>
</tr>
</tbody>
</table>

13.3.1. Ions and small molecules

Early attempts at inhibiting the TCS regulon provided encouraging results. A new class of antibacterials, hydrophobic tyramines, that inhibits the first component of TCS, histidine kinase, has been discovered. Notable representatives are RWJ-49815 and its analogs. They inhibited bacterial growth and expressed reduced emergence of resistance in laboratory passage experiments [77].

Some 2-aminoimidazole-containing compounds that inhibit VraSR TCS were shown to suppress resistance to beta-lactam antibiotics including oxacillin and carbapenems [78].

As mentioned in the stress response section above, sub-lethal metal stresses can induce AMR. However, it has been shown that Zn or Mg at optimal concentrations can significantly increase the activity of antibiotics (9–15 µg/antibiotic disc; 3–9 µg/antibiotic disc) [79]. Recently, data indicated that Zn at 0.2 mM, for example, can inhibit the SOS response and hypermutation phenomenon in bacteria via interference with the actions of RecA and
protecting LexA, an initiator of the SOS response, from RecA-mediated cleavage, thereby reducing the rate and magnitude of AMR [80].

Recognising the importance of RecA in AMR development, a study in 2007 screened multiple small molecules for RecA inhibition activity. It found that curcumin and polysulfated naphthyl compounds, including congo red, suramin, and bis-ANS, substantially inhibited RecA’s ATPase activity, among which the polysulfated naphthyl compounds seemed to have an effect on ATP hydrolysis activity via direct binding to RecA while curcumin did not [81].

Curcumin, a natural polyphenolic flavonoid derived from Curcuma longa L. or turmeric, is known for various biological activities including anti-inflammatory, antioxidant, antibacterial, and wound-healing accelerating activity. The average minimum inhibitory concentration (MIC) values of curcumin against Gram-negative and Gram-positive bacteria were 117.4 mg L\(^{-1}\) and 126.9 mg L\(^{-1}\), respectively [82]. Recent evidence suggested that curcumin at 8 mg L\(^{-1}\) can inhibit the SOS response in E. coli induced by levofloxacin or ultraviolet (UV) irradiation [83,84]. The exact mechanism is still unclear but it is suggested that curcumin might have a role in preventing the binding of ssDNA to RecA protein, therefore lowering the SOS response [84]. Furthermore, it was shown that curcumin can trigger a synergistic effect when used together with beta-lactam and quinolone antibiotics, among which ciprofloxacin had maximum synergy for Gram-positive and amikacin, gentamicin, and cefepime for Gram-negative isolates [82]. In addition, it also restored sensitivity of methicillin-resistant Staphylococcus aureus (MRSA) to antibiotics and its antibacterial effect increased markedly when used together with membrane permeability enhancers and ATPase inhibitors [83]. In conclusion, with low toxicity curcumin is suggested to have a beneficial role in supplementing antibiotic therapy.

Suramin (Germanin), a polysulphonated naphthyl urea, inhibits DNA strand exchange, ATPase activities of bacterial RecA, and RecA-catalysed proteolytic cleavage of the LexA repressor. Data showed that suramin abolished ciprofloxacin-induced recA gene expression and the SOS response and augmented the bactericidal action of ciprofloxacin [86]. Suramin and polysulfated naphthyl compounds, though previously considered to have little therapeutic utility due to their negative charge causing difficulty in crossing cell membranes, have been used to treat parasitic infections (filariasis, trypanosomiasis) and viral infections as well as maglinancies. It is possible that suramin and its derivatives could be developed into adjuvants for antibiotic chemotherapy, which can reduce AMR development.

A high-throughput screening method for RecA/LexA axis inhibitors has recently been developed. It has screened more than 1.8 million compounds and identified new small molecule classes that target the LexA autoproteolysis step in SOS activation. Among those newly-identified compounds, C1 and D1 (Figure 2) showed remarkable inhibition activity [87].
**Figure 2.** Chemical structure of organic compounds with potential to inhibit the bacterial stress response

A) Suramin

B) C1

C) D1

D) TCS inhibitor RWJ-49815

E) 2-aminimidazole derivatives
13.3.2. Polyamines and peptides

Polyamines are organic cationic compounds with two or more primary amino groups. The low-molecular-weight linear polyamines, such as putrescine, spermidine, spermine, and cadaverine, are present in most organisms and perform essential functions including cell growth, survival, proliferation, and stress responses, such as oxidative or antimicrobial stress. Oxidative stress, for example, was reduced via upregulation of oxyR and katG under the effect of either endogenous or exogenous polyamines such as putrescine [88]. Some research also indicated that polyamines affected bacterial adaptations to stress with high involvement of the RpoS pathway [62]. The polyamines cadaverine, putrescine, and spermidine increased rpoS expression, which can result in the reduction of porin permeability as a response to prevent antibiotic penetration into the cell, thus leading to AMR. Exogenous polyamines, however, differently affected antimicrobial susceptibility. While MICs of beta-lactam antibiotics were markedly reduced in the presence of spermine and spermidine, for example 200 folds for oxacillin, the MICs of polymyxin and ciprofloxacin were increased. Synergistic effects of polyamines with antibiotics were proven not to be related to porin and efflux pumps in some cases; however, detailed mechanisms are not yet clear [92].

A recent finding showed that a synthetic peptide, DJK-5, was able to interfere with bacterial stress responses and heal abscesses in mice [91]. This small cationic peptide consisting of d-aminoacids caused rapid degradation of the stringent response mediator, alarmone guanosine tetraphosphate (p)ppGp, resulting in stringent response inhibition. It also had strong antibiofilm activity against different multi-drug resistant Gram-negative bacteria [91]. Similar to DJK-5, synthetic peptide 1018 was also proven to target the same pathway with high involvement to SpoT and RelA. Together, they offer a promising novel therapeutic approach in fighting AMR [90,93].

Not surprisingly, scientists have developed peptide inhibitors of RecA/LexA, undebatably important targets to inhibit SOS response, which is a principal mechanism of bacterial adaptation to antibiotics. According to the sequence of RecX protein, an active natural RecA inhibitor, 20-amino acid alpha helical peptides were designed showing promising in vitro RecA inhibiting activity and in vivo SOS response blocking activity [89].

Lately, developing inhibitors of up-stream regulatory molecules in the stress response network, such as sigma factors and regulatory RNAs, have attracted research interest and produced a few pioneering results. Inhibitors of sigE, for example, have been investigated via high-throughput screening with small cyclic peptides generated in E. coli by using split-intein circular ligation of proteins and peptides (SICLOPPS), a genetic system based on spontaneous protein splicing by inteins. Results showed that some cyclic peptides expressed impressive sigE inhibition activity in vitro but not yet in vivo when added exogenously into bacterial culture as an antibiotic. This is probably due to the
inability of these cyclic peptides to cross cell envelopes, leading to low intracellular accumulation [94]. The same research group also attempted to find inhibitors for the Hfq-sRNA system, and they found one cyclic peptide, RI20, that is able to inhibit gene expression of Hfq-mediated regulation in cooperation with RybB and MicF [95]. Interestingly, under the presence of RI20, bacterial cells become more susceptible to oxidative stress (challenged with hydrogen peroxide) and antibiotics such as benzalkonium chloride and novobiocin [94].

13.4. CONCLUDING REMARKS

Understanding the bacterial stress response creates opportunities to efficiently use existing antibiotics as well as better understand their mechanisms of action. Preliminary success in interfering with stress response pathways brings hope to antibiotic co-therapy, which can enhance antibiotic efficacy and reduce antibiotic resistance development. Recent approaches to develop multiple-target inhibitors or inhibitors of upstream regulators of antimicrobial and antimicrobial-related stress response can reduce the effect of target mutation or systemically impair bacterial physiology. Future research will show whether we can widely apply this approach in clinical settings.

REFERENCES


Chapter 14

BACTERIAL ADHESION ON POLYELECTROLYTE MULTILAYERS

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14.1. INTRODUCTION

In medicine, the interactions between microorganisms and material surfaces are of fundamental importance. In nature, bacteria can be present as planktonic cells, which are freely moving in a bulk solution [1]. The attractive interactions between microorganisms and surfaces can lead to bacterial adhesion, which is the first step in microbial colonization and biofilm formation. The biofilm has been defined as a complex, three-dimensional functional society of adherent microorganisms bound to, and growing at, an interface and encased by an extracellular polymeric matrix [2,3]. The adhesion process is the result of different physical and chemical processes. Various parameters, such as the properties of a microbial cell (cell surface hydrophobicity and charge, extracellular appendages, extracellular polymeric substances, signalling molecules), fluid (polarity, flow velocity, pH, ionic strength, temperature, presence of salts, antimicrobials, nutrient availability), and surface chemistry (hydrophobicity, electric charge, surface roughness, etc.) can influence the bacterial adhesion [4].

Biofilms are clusters of microorganisms that are almost always found with healthcare-associated infections (involving medical devices, such as catheters, implants, pacemakers, and prosthetics). Vascular catheter-related bloodstream infections are the most serious infections [5]. Urinary catheters are made of tubular latex or silicone devices, and if inserted into the human body they may readily acquire biofilms on the inner or outer surfaces. With increasing time, the urinary catheter can become a place where microorganisms develop biofilms, resulting in urinary tract infections. Medical implants can be the location of device-related infections, which are difficult to eradicate because bacteria-causing infections live in well-developed biofilms [6]. The implants that can be compromised by biofilm associated infections are the following: heart valves, central venous catheters, ventricular assist devices, coronary stents, neurosurgical ventricular shunts, implantable neurological stimulators, fracture-fixation devices, arthro-prostheses, inflatable penile implants, breast implants, cochlear implants, intraocular lenses, and dental implants [7]. Biofilms on the previously mentioned medical devices are composed of gram-positive or gram-negative bacteria or yeasts. Bacteria usually isolated from medical devices include the gram-positive Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, and Streptococcus viridans and the gram-negative Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa. These organisms usually originate from the skin of patients or workers in the health-care system [6]. Detachment of cells, production of endotoxin, increased resistance to the host immune system, and provision of a niche for the generation of resistant organisms can all result in biofilm production that may initiate an infection [8].
Biofilms also play an important role in antimicrobial-drug resistance. Bacteria present in biofilms are more resistant to antimicrobial agents than planktonic cells. The reason is the diminished rates of mass transport of antimicrobial molecules to the biofilm associated cells. On the other hand, the biofilm cells usually differ physiologically from planktonic cells [6].

Recently, many attempts have been made to develop coatings of implantable devices to reduce the bacterial adhesion and biofilm formation. A special, but very important, case of possible biomedical applications is the development of antibacterial polyelectrolyte multilayers (PEMs).

14.2. POLYELECTROLYTE MULTILAYERS

It is well-known that by mixing two aqueous solutions of positively and negatively charged polyelectrolytes that the aggregates form predominantly due to the electrostatic attraction between the oppositely charged chains. Such aggregates are generally known as polyelectrolyte complexes or interpolyelectrolyte complexes, although in early work, the terms complex flocculation and complex coacervation were also used. Stability of such complexes can be, in addition to predominant Coulombic forces, influenced by hydrogen bonds and hydrophobic interactions, etc. Structure and characteristics of such aggregates are determined by different factors, such as the nature of ionic groups, initial polyelectrolyte concentrations, pH, ionic strength, temperature, and preparation procedure.

Among the first investigations in this field, the efforts of Bungenberg de Jong [9] and Kruyt [10] should be mentioned. They were following the formation and structure of polyelectrolyte complexes, at that time called complex coacervates. They investigated the influence of pH and molar mass of polycations and polyanions on the complex stability, as well as the condition of polyelectrolyte complex formation. Overbeek and Voorn [11] have tried to theoretically explain the experimental results obtained by Bungenberg de Jong and Kruyt. Using the Debye-Hückel theory, they determined the electrostatic energy and the entropy of mixing for two-component (polyelectrolytes and solvent) and three-component (polyelectrolytes, solvent, and inert electrolyte) systems. These early efforts in the field of polyelectrolyte complexes were followed by the Michaels’ [12] studies of complexes formed by synthetic polyelectrolytes with high charge density. Later on, Dautzenberg [13,14] investigated the structure and the characteristics of strong polyelectrolytes formed by sodium polystyrene sulfonate and poly(diallyldimethylammonium chloride) and their acrylamide copolymers. Using different experimental methods, he managed to determine the complex stoichiometry, the amount of bound sulfonate groups, the ratio of polycationic and polyanionic sites in the complex, as well as mass, size, and structural density of the complex. He
showed that the presence of a small amount of salt during complex formation causes much lower values of the degree of aggregation. It is known that, especially for systems containing at least one weakly charged component, the type of supporting electrolyte and ionic strength influence the formation of aggregates. It was additionally shown by Dautzenberg and Karibyants [15] that complexes formed by strong polyanions (sodium polystyrene sulfonate) and strong polycations (poly(diallyldimethylammonium chloride)), as well as the complexes formed by weak polyanions (sodium polymethacrylate) and strong polycations (poly(diallyldimethylammonium chloride)) show in the presence of an inert electrolyte a lower degree of aggregation then in the absence of an inert electrolyte. The increase in ionic strength in such a system leads to secondary aggregation. Additionally, systems containing weak polyelectrolytes show a lower degree of aggregation in comparison with the systems containing strong polyelectrolytes. Numerous results in this field were obtained by Kabanov and his coworkers [16–20]. They investigated the formation, structure, and characteristics of different polyelectrolyte complexes obtained by mixing both strong and weak polyanions with strong polycations. The influence of ionic strength on the polyelectrolyte complex formation was thoroughly investigated by Pergushov et al. [21]. They used the fluorescence quenching method to explore the effect of different salts and the degree of polymerization on the nonstoichiometric complex formation.

Since 1991, when Decher introduced the layer-by-layer method of constructing PEMs on metal oxide surfaces [22], interest in the process of formation of such layered structures has been continuously growing.

![Figure 1. Scheme of PEMs having a negative (left) and a positive (right) terminating layer formed on a silica surface](image)

Various experimental methods [23,24] applied to obtain a better insight into the process has helped increase the understanding of the multilayer formation process. The ease of formation of multilayers motivated scientists to extend the type of constituents incorporated into such nanocomposites by including proteins [25], dendrimers [26], and even DNA [27,28].
As stated above, the layer-by-layer deposition method to prepare multilayers of polyelectrolytes of alternating charge can be followed by various experimental methods. Very interesting results were obtained by stagnation point optical reflectometry [29,30], ellipsometry [25], quartz crystal microbalance [31], and optical waveguide lightmode spectroscopy [32]. Here, we will concentrate on the cases where different effects have been followed in situ by means of optical reflectometry experiments [29,30,33,34]. It turns out that in solutions containing both polyelectrolyte and appropriate salts up to a certain concentration, the regular build-up of multilayers is modified and becomes an adsorption/redissolution process [28]. This was explained by taking into account (i) that during the regular multilayer formation process the macromolecules cannot equilibrate, (ii) that the added salt plasticizes the multilayer to a state where the molecules are sufficiently mobile to enable them to equilibrate between the layer and the surrounding solution, and (iii) that the presence of excess polyelectrolyte brings the system to a one-phase region of the polyelectrolyte complex phase diagram, implying that polyelectrolyte complexes must dissolve under these conditions.

Additionally, the influence of different salts (phosphates, chlorides, and nitrates) and polyelectrolyte molecular weight on the formation and erosion of multilayers on silica surfaces was also investigated by means of the optical reflectometry method [30]. In all of these experiments, the anionic polyelectrolyte was poly(acrylic acid). On the other side, three different cationic polyelectrolytes were used: poly(dimethylaminoethyl)methacrylate, poly(allylamine hydrochloride), and poly(2-vinyl-N-methylpyridinium iodide). It has been shown that at very low ionic strength (1 mM) regular build-up of multilayers is observed independent of the salt used. However, at higher ionic strength, dissolution also takes place, and the critical "glass transition ionic strength" needed for the multilayer to be dissolved depends on the salt used, as well as on the polycation/polyanion pair studied.

The application of optical reflectometry for the characterization of multilayers obtained by exposing a suitable substrate to solutions of a cationic homopolymer and an oppositely charged protein (which could also be of interest for studying bacterial adhesion) was also performed [33]. In these experiments, the negatively charged component bovine serum albumin (BSA) was used. As the cationic homopolymer, a weak polyelectrolyte poly(dimethylaminoethyl)methacrylate (PAMA) or a strong polyelectrolyte poly(2-vinyl-N-methylpyridinium iodide) (PVP+) were used. As the solid substrate, we used silica in the form of silicon wafers carrying an oxide layer, and as the supporting electrolyte we used potassium chloride or phosphate buffer. The influence of ionic strength, pH, type of cationic homopolymer (strong or weak), and protein concentration were investigated, and it was shown that stagnation point optical reflectometry can be a useful method for characterization of the formation of multilayers containing polyelectrolyte and protein layers. The method of stagnation point optical reflectometry was also
applied for examining the adsorption of BSA on the previously formed poly(allylamine hydrochloride)/poly(sodium 4-styrenesulphonate) (PAH/PSS) multilayer, with PAH being a terminal layer [34]. The solid substrate was silica in the form of silicon wafers carrying an oxide layer. In order to interpret the adsorption of BSA, the build-up mechanism of the PAH/PSS multilayers was examined, with special emphasis on the effect of electrolyte concentration, pH of solution, and the anchoring (precursor) layer on that process. Additionally, the effect of BSA concentration and the anchoring layer on BSA adsorption was investigated. It was shown that in all investigated systems, the adsorption of BSA depends on the conditions under which the multilayer was formed (ionic strength, pH, and presence of an anchoring layer), as well as on BSA concentration. It follows that the adsorption of BSA could be controlled not only by choosing suitable BSA concentration, but also by modifying the preformed multilayer.

Since layer-by-layer structures (e.g. PEMs and protein-PEMs) play a very important role in surface modification processes, it was intriguing to check if other experimental methods would confirm the results and conclusions obtained by stagnation point optical reflectometry. Therefore, electrokinetic measurements were also applied for the investigation of PAH/PSS multilayer formation as a function of pH, with PAH being a terminal layer [35]. The instrument uses electrophoretic light scattering and the Laser Doppler Velocimetry method for determination of particle velocity, and from this the electrokinetic zeta potential can be determined. The electrokinetic potential was calculated from mobility values using the Smoluchowski equation. Additionally, the effect of supporting electrolyte (KCl) concentration on multilayer formation was tested. Silica particles were used as the solid substrate. The adsorption of BSA on the previously formed multilayer was examined as a function of pH and BSA concentration. The experiments were performed at three different ionic strength values as a function of pH, and it was confirmed that the electrokinetic measurements were suitable for monitoring the formation of various multilayers. In all investigated systems, the process of multilayer formation was found to depend on conditions (ionic strength and pH) under which the multilayer was formed. Moreover, BSA concentration also played a significant role in the adsorption on the previously formed multilayer.

- All the above mentioned results (both the results that concern polyelectrolyte complexes as well as the ones that concern PEMs) were shown to depend on so-called ionic conditions. That means that the formation processes are very sensible to the concentration of added supporting electrolyte, but also to the electrolyte type. In order to obtain a deeper insight into effects occurring when electrolyte solution is added to solution of a strong polyelectrolyte, the microcalorimetric and potentiometric titrations of poly(sodium 4-styrenesulfonate) (Na⁺PSS⁻) solution with different alkali, earth-alkali, and
tetraalkylammonium nitrate, perchlorate, and chloride solutions were performed [36]. From the calorimetric titrations, the differences in sign and magnitude of enthalpy change upon addition of various electrolytes were observed depending on the salt used. Potentiometric titrations using a sodium ion-selective electrode have revealed that addition of electrolyte is accompanied by the increase in sodium activity until a certain critical value is reached, which seems to be the consequence of counterion substitution on the polyelectrolyte chain. In the case of the addition of lithium and sodium salts, the experimental results for the change in enthalpy of mixing (ΔH) can be qualitatively correctly explained by the Poisson–Boltzmann and Monte Carlo calculations based on the continuum solvent models. This is not the case for the mixtures with KNO₃, RbNO₃, and CsNO₃ salts. The results suggest that the ion-specific effects, associated with the changes in water structure, have to be taken into account when thermodynamic properties of polyelectrolytes in solution are concerned. The calorimetric results imply that the enthalpically observed cation specificity for binding to the poly(styrenesulfonate) group could be correlated with the corresponding cation hydration enthalpies. The counterion substitution of sodium with divalent cations was found to be endothermic, which is in qualitative agreement with the electrostatic theory.

14.3. APPLICATION OF POLYELECTROLYTE MULTILAYERS FOR STUDYING BACTERIAL ADHESION

The adsorption of biological or biomimetic structures onto certain synthetic materials (in this case PEMs) could enable additional progress in the field of biosensing surfaces, tissue engineering, and drug delivery. PEMs formed by alternate adsorption of positively and negatively charged polyelectrolytes are promising coatings onto which biological molecules (e.g. proteins) could be adsorbed. In the literature [37–39], several examples of investigation of adsorption of different proteins on previously formed multilayers using different experimental methods can be found. Müller and coworkers [37] examined the sorption of human serum albumin (HSA) on poly(ethyleneimine)/poly(acrylic acid) multilayers using attenuated total reflection Fourier transform infrared (ATR FTIR) spectroscopy, while Gergely et al. [38] analysed adsorption of the same protein on poly(L-lysine)/poly(glutamic acid) multilayer by Optical Waveguide Light-Mode Spectroscopy (OWLS) and atomic force microscopy (AFM). The secondary structure of BSA adsorbed onto PAH/PSS multilayers was also investigated by
Schaaf and coworkers [39], and it was observed that PAH as the terminal layer has practically no effect on aggregation of BSA.

A special and very promising case is the possible application of PEMs as antibacterial coatings [40–44]. Many investigated PEMs promote or disrupt bacterial biofilm formation simply because of their high surface charge density. If the protruding chains bear an opposite charge than the bacteria, then these become attached to the PEM surfaces. In the case of their like charge, the adhesion is hindered. Zan and Shu [40] proposed a scheme for the procedure for fabricating PEMs containing silver ions or silver nanoparticles, which could be used as effective antibacterial coatings. On the other hand, Wong and coworkers showed that protein adsorption is drastically lowered on microbicidal hydrophobic/hydrophilic PEMs [41].

In addition to charge, factors that could affect the intensity of bacterial adhesion include material surface roughness, charge, degree of hydrophobicity, Lewis acid-base character, and hydrogen-bonding capacity [45-48]. Environmental factors, including pH, temperature, nutrient composition, and population characteristics may enhance the adhesion and biofilm maturation [48].

Taking into account that PEM-modified surfaces could exhibit significantly improved bacterial anti-adhesive properties, it is important to relate the conditions (polyelectrolyte concentration, salt concentration and type) at which the PEMs are formed, the number of polyelectrolyte layers, and the type of terminating layer with the corresponding adhesion of bacteria.
14.4. BACTERIAL ADHESION MEASUREMENTS

14.4.1. Key adhesion parameters

As stated above, to study bacterial adhesion rate we need to first characterize the material (in this case PEM) and the bacterial surfaces to determine the key parameters in the adhesion process. For example, one of these parameters is surface roughness, which could be measured with AFM or profilometry. Surface hydrophobicity can be obtained from contact angle measurements, while the surface charge can be determined from electrophoretic or streaming potential measurements. On the other hand, many methods for adhered bacteria counting have been published, including direct counting methods, such as scanning electron microscopy (SEM), and indirect counting methods, such as colony forming units (CFU), plate count, and staining methods [49,50].

![AFM picture of metal surface AISI 304 3C. The root mean square (RMS) roughness is 160.5 nm. Right: SEM picture of adhered bacteria on electropolished surfaces after 24 h.]

**Figure 2.** Left: AFM picture of metal surface AISI 304 3C. The root mean square (RMS) roughness is 160.5 nm. Right: SEM picture of adhered bacteria on electropolished surfaces after 24 h.

14.4.1.1 Surface charge influence

As stated above, one of the key factors that influences the intensity of bacterial adhesion is the charge of the surface. For that purpose, zeta-potential measurements can be used. In our earlier study [51], we applied zeta-potential measurements for studying the properties of the PEMs formed from poly(allylamine hydrochloride), PAH, and sodium poly(4-styrenesulfonate), PSS. These synthetic polyelectrolytes have been widely used in the process of PEM formation, and we also used them extensively in our investigations of polyelectrolyte complexes [52]. The example of the change in surface charge of PEMs (i.e. the change in zeta-potentials) is presented in Figure 3 for the formation of PAH/PSS multilayers.
Figure 3. The dependence of the zeta-potential on the number of oppositely charged polyelectrolyte layers in the PAH/PSS system in the presence of sodium perchlorate; 
\[ c_m(\text{PAH}) = c_m(\text{PSS}) = 0.001 \text{ mol dm}^{-3}, \quad c(\text{NaClO}_4) = 0.001 \text{ mol dm}^{-3}, \quad t = 25 ^\circ\text{C}. \]

We showed [51] that polyelectrolyte concentration, as well as supporting electrolyte (added salt) concentration, significantly influence the multilayer build-up. Therefore, in the process of PEM formation, special attention should be given to controlling these experimental parameters. Of course, other experimental conditions which are known to influence PEM build-up, such as pH, polyelectrolyte type, added supporting electrolyte type, molecular weight, and temperature should be also taken into account.

The importance of charge for bacterial adhesion was also shown by Lienkamp and coworkers [53]. They prepared multilayers formed from poly(acrylic acid) (PAA) and either the hydrophobic butyl synthetic mimics of antimicrobial peptides (SMAMP) or the hydrophilic diamine SMAMP with a poly(ethylenimine) anchoring layer, and found that the positive charge of PEMs with SMAMP as the terminating layer is low, since a significant part is consumed to maintain layer stability. This leads to reduced antimicrobial activity. The suppression of biofilm formation can also be achieved by introducing strongly hydrophilic groups at the surface of PEMs. However, the prevention of bacterial adhesion cannot result in their complete elimination. This can be achieved once stable bacteria attachment is achieved.
14.4.1.2 Influence of other surface parameters

As stated above, other key parameters, such as surface hydrophobicity and surface roughness, should be examined. In the case of PEMs formed on oxidized silicon wafers prior to adhesion of bacteria, the obtained hydrophobicity (i.e. contact angle) was similar on PEMs terminating with a polycation and with a polyanion layer. In the first case, the contact angle was $48.9^\circ \pm 2.5^\circ$ and in the latter one the contact angle was $46.9^\circ \pm 5.0^\circ$.

The results of roughness measurements obtained by means of atomic force microscopy did not differ much in the case of the two types of multilayers. In the case of the positive surface (polycation terminating multilayer), roughness was determined to be $0.017 \mu m \pm 0.004 \mu m$, and in the case of the negative surface (polyanion terminating multilayer), roughness was $0.019 \mu m \pm 0.006 \mu m$.

<table>
<thead>
<tr>
<th>Table 1. PEMs with terminating layers bearing positive or negative charge investigated in terms of the contact angle value and roughness. Adapted from [51].</th>
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<tr>
<td>Positively charged polyelectrolyte PAH as terminating layer (5 layers)</td>
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<tr>
<td>Contact angle</td>
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<td>Roughness</td>
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14.4.2 Adhesion of Pseudomonas aeruginosa

For examining the adhesion of bacteria, *P. aeruginosa* is very often used. *P. aeruginosa* is a common pathogenic bacterium with a possible multidrug resistance by mutation and could be responsible for various possible postoperative infections. Recently, Dekany and coworkers showed [54] that clinically relevant pathogen strains (for example, *P. aeruginosa*) could be inactivated by the photocatalytically active titanium dioxide functionalized with silver nanoparticles and immobilized in polyacrylate-based nanohybrid thin film [55]. In our study [51], we showed that the fraction of the multilayer surface covered with *P. aeruginosa* was $20.4\% \pm 4.8\%$, with PAH as the terminating layer, and $9.0\% \pm 3.1\%$, with PSS as terminating layer. These results allowed the assumption that differences in bacterial adhesion capability between the systems with oppositely charged terminating layers should be the result of electrostatic interactions. That is in accordance with predictions since the bacteria cell walls possess negative charges. Bohinc *et al.* [46] measured the zeta potential of *P. aeruginosa* for two phosphate buffer solutions at two ionic strengths, $1 \text{mmoll}^{-1}$ and $100 \text{mmoll}^{-1}$, and the negative
zeta potentials were obtained as follows: $-16.92 \text{ mV} \pm 2.42 \text{ mV}$ for 1 mmol l$^{-1}$ and $-7.85 \text{ mV} \pm 12.8 \text{ mV}$ for 100 mmol l$^{-1}$.

![Figure 4. Schematic presentation of the adhesion of bacteria on PEMs with negative (left) and positive (right) terminating layers [51]](image-url)

14.5. PERSPECTIVES

In recent years, significant progress in the design of surface coatings has been made [43]. Generally, there are two possibilities for preventing bacterial adhesion to surfaces and subsequent bacterial infections, which are physical and pharmacological. PEMs can be used for both attempts. They can be applied as antifouling coatings in which antibacterial substances, such as antibiotics, can be incorporated. Antibacterial substances are then slowly released.

In the existing antimicrobial implant coatings, drug dosage and release rate cannot be easily tuned. Local sustained delivery is still difficult to achieve. For these problems, specific PEMs [51] can improve the efficiency and tunability of the drug dosage [56].

In many cases, the initial contamination of medical devices is mostly caused by a small number of microorganisms. They are often transferred to the device via the patient’s or healthcare workers’ skin, contaminated water, or other external environmental sources. Infections in medicine cause a huge financial burden on healthcare services. On the other hand, infections are responsible for the patient’s morbidity and mortality. To solve the mentioned problems, PEMs on implantable devices are especially attractive.

14.5. CONCLUSIONS

In this chapter, we considered the bacterial adhesion on material surfaces coated with PEMs. Generally, bacterial adhesion can be controlled by different material and bacterial surface properties, such as surface roughness, surface...
charge, hydrophobicity, and specific surface structure. It is important to state here that the combination of surface characterization with microbial testing leads to the better understanding of the bacteria-surface interactions. Regarding surfaces coated with PEMs, it seems that the main factor for bacterial adhesion is the surface charge. In terms of PEM formation, adjusting salt type and concentration, as well as the appropriate terminating polyelectrolyte layer, can lead to the formation of multilayer systems with optimized antibacterial properties.

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Chapter

Chapter 15

IMMUNOMODULATION BY ANTIBIOTICS

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15.1. INTRODUCTION

Since more and more bacteria are developing resistance against the direct impact of antibiotics, it is important to consider the broader effects of these drugs for strategies to combat resistance. Several antibacterials are able to exert indirect antibacterial actions that can be utilized for this purpose. The modulation of the immune system, as part of the host defense response, can boost antibacterial defense and facilitate clearance of bacteria [1]. In this connection, immunomodulation by several antibiotics, particularly macrolides but also quinolones, can either promote bacterial killing or facilitate resolution of inflammation and reduce bystander tissue injury. Some of these antibiotics can modify bacterial inflammation even when resistance to the primary effects of the drugs occur. In some cases, these antibiotics are even effective against respiratory viral infections by reducing the cytokine storm [2]. Incorporation of immunomodulation into the activity profile of antibiotics can thus, expand their breadth of activity in bacterial infections, independent of the degree of bacterial resistance [3].

Most infections are accompanied by inflammation which is characterized by increased accumulation of inflammatory cells (monocytes, macrophages, granulocytes, plasma cells, lymphocytes and platelets). Together with the tissue endothelial cells and fibroblasts, these cells release a variety of enzymes, cytokines, growth factors and lipid mediators which can lead to tissue damage. Tissue injury is dependent on the type of immune response. For example, neutrophil-dominated inflammatory diseases that include bacterial infections as well as active phases of rheumatoid arthritis, cystic fibrosis, bronchitis and chronic obstructive pulmonary disease, are characterized by the release, among other products, of proteinases and reactive oxygen species (ROS) by neutrophils which also exert antibacterial responses [4].

Neutrophils are an essential component of the host defense system against bacterial infections. It has become clear that the modulation of neutrophil activation is a promising approach for the regulation of inflammatory conditions [4]. In their role as a decisive defense force, neutrophils have developed several mechanisms of antibacterial activities. First of all, the phagocytically triggered respiratory burst is an effective way of destroying invading microorganisms. Thereby, a huge quantity of toxic ROS (e.g. superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide, hypohalous acid) are generated. Secondly, neutrophils generate secretory granules which contain effective degradative enzymes and bactericidal proteins (e.g. myeloperoxidase, elastase, cathepsin, collagenase, neuraminidase, heparanase, defensins). There is a risk, however, that these defensive mechanisms might not only kill the invading bacteria, but also harm the host tissue. Consequently, resolution of this acute inflammatory response is
essential to avoid unintended tissue damage [5]. Locally released lipids like prostaglandin D2 derivatives exert an important role in the resolution of the inflammatory state by promoting apoptosis in neutrophils [6]. The programmed cell death of neutrophils provokes reduced release of pro-inflammatory cytokines [7,8]. During the progress of inflammatory resolution, macrophages play a crucial role through their phagocytosis of apoptotic neutrophils. Furthermore, macrophages support the tissue healing process by releasing growth factors, removing tissue debris and stimulating formation of replacement connective tissue [8]. The balance between the initiation of inflammation which results in the killing of the pathogens and the well-timed resolution of the inflammation to prevent tissue damage is a complex mechanism which can be modulated by several antibacterials [1,3]. The immunomodulating effects of antibacterials can either promote the initial antibacterial defense and/or the subsequent process of inflammatory resolution. Therefore, an important factor in the pharmacological modulation of inflammation is the timing of the treatment. Stimulation of the acute inflammatory response, e.g. by additional activation of neutrophils, promotes the removal of the bacteria and boosts thereby, the host defense. At a later time point, the stimulation of leukocyte apoptosis and the reduction of released pro-inflammatory cytokines is important to prevent tissue damage in infectious diseases and non-infectious chronic inflammatory conditions. Especially in patients with chronic inflammatory disorders, the anti-inflammatory properties of antibacterials and other drugs are implemented to limit ongoing tissue damage (Figure 1).

The particular benefits of these immunomodulatory effects include promotion of host defense, even when bacterial resistance may dampen sensitivity to the direct impact of the antibiotics. The relevant phases of the inflammatory process and the opportunity to modulating these phases with antibacterial agents will be described in the following section.
Figure 1. Overview of the main actions of antibiotics in the three main phases of inflammation. Stimulatory actions are indicated by unbroken lines while inhibitory actions are denoted in dashed lines. Antibiotics are indicated by italic letters. The initiation phase consists of vasodilation, adhesion, chemotaxis, transendothelial migration of leukocytes and plasma exudation. Several antibiotics show inhibitory actions during this phase while erythromycin has stimulatory effects on endothelial cells and NO production. During propagation, the inflammatory leukocytes are activated and inflammatory mediators, as well as degradative enzymes, are released. This leads to the destruction of the invading pathogens. Plasma exudation is further promoted and tissue damage near the inflammation side occurs. In this phase, antibiotics have mainly inhibitory effects. Macrolides (azithromycin) and quinolones (minocycline) exhibit stimulatory actions in the initiation and the early phase of propagation. Inflammatory resolution is associated with enhanced apoptosis and the release of anti-inflammatory cytokines. Apoptosis is promoted by macrolides but inhibited by several other antibiotics. MMP = metalloproteinase, NO = nitric oxid.
15.2. THE INFLAMMATORY PROCESS AND INTERACTIONS WITH ANTIBACTERIALS

Several antibacterials show inflammation modulating effects on leukocytes, especially on neutrophils. Among others, macrolides, ketolides, quinolones and tetracyclines show the most pronounced effects and are reviewed here with regard to their immunomodulatory properties.

15.2.1. Accumulation in inflammatory cells

To be able to exert immunomodulating effects, antibacterials must first accumulate in the immune cells. There is evidence that macrolides reach concentrations of up to several hundred-fold higher in inflammatory cells in comparison to the extracellular fluid [4,9]. This results in effective delivery of the antibacterials to the site of inflammation/infection. The exact mechanism by which this intracellular accumulation of macrolides is achieved is yet unclear, but there are hints that it is an active protein-mediated process [10]. Accumulation takes place particularly in the cytoplasm and the azurophilic granules of neutrophils, resulting in effective delivery of the macrolides to phagocytosed bacteria. Cytokines enhance macrolide accumulation in macrophages in vitro, so that even more pronounced accumulation of macrolides at the site of inflammation/infection might be observed under inflammatory conditions in vivo [11].

There are several differences with respect to the efflux of the various macrolides. Clarithromycin and erythromycin show a very fast release from leukocytes while azithromycin remains longer within the cells [9,12,13]. The prolonged residence of azithromycin facilitates a longer antibacterial effect and a pronounced immunomodulating effect which might not be observed in short-term cell culture experiments in vitro.

Macrolides show the most distinct accumulation in immune cells, but other antibacterials also show similar effects. For example, clindamycin shows an approximate 20-fold intracellular accumulation within alveolar macrophages via the nucleoside transport system [14]. Chloramphenicol, lincomycin, tetracycline and rifampin also show selective accumulation by about 2- to 5-fold. Within neutrophils, the concentrations of ciprofloxacin and quinolones are about 5-fold higher than in the extracellular fluid [15]. After accumulation in the immune cells, the antibacterials can modulate the further steps of the immune response.
15.2.2. Effects on immune cell infiltration and vasodilation

One of the first essential steps in the inflammatory process is the infiltration of neutrophils and further immune cells into the tissue. This recruitment includes a sequence of events which involves the specific arrest of the immune cells on the vascular endothelium and their transmigration across the endothelial cell barrier (Figure 1). The adhesion process is characterized by four phases (margination, capture, rolling and adhesion) which are mediated by several cell adhesion molecules of the integrin and selectin family. The expression of these adhesion molecules is stimulated by pro-inflammatory cytokines [16]. Furthermore, chemokines and complement anaphylatoxins influence the directed migration of the immune cells. Vasodilatory factors such as prostaglandin E2 lead to plasma exudation and swelling which also affect the infiltration process. These initial steps in the process of inflammatory response are modified by several antibacterials [1,3,17].

An important molecule for the adhesion process is Mac-1 (CD11b/CD18). It has been shown in patients with diffuse panbronchiolitis (DPB) that Mac-1 is upregulated on peripheral neutrophils compared to healthy subjects [18]. The number of infiltrating immune cells at the site of inflammation is reduced by inhibition of these adhesion molecules. Several macrolides have an impact on the adhesion molecules of neutrophils. Roxithromycin is able to reduce the expression of Mac-1 on peripheral neutrophils from patient with DPB. Treatment for 2 weeks with erythromycin inhibits expression of Mac-1 and L-selectin which results in a reduced infiltration of macrophages and neutrophils into the site of inflammation in rats with experimental otitis media [19]. Additionally, erythromycin reduced L-selectin expression in an LPS-induced neutrophil recruiting model in rats [20]. Both roxithromycin and erythromycin decrease intercellular adhesion molecule-1 (ICAM-1) expression, which is a surface protein of the integrin family [21,22]. Roxithromycin was ineffective on whole blood cells in experiments in vitro, while a reduction of Mac-1 expression on neutrophils was observed after treatment of patients with lower respiratory tract disease like DPB [18]. This indicates that prolonged treatment is necessary for the inhibitory effects. That delayed action has the advantage that at the beginning of infection and of treatment, when the immune response is needed, the macrolides did not reduce the infiltration of immune cells. Later on, when the immune response switches to resolution, the macrolides promote the process by preventing further immune cell migration into the tissue. Furthermore, roxithromycin was able to inhibit adhesion of neutrophils to epithelial cells in vitro [21]. Clarithromycin reduced the expression of several adhesion molecules, like vascular cell adhesion molecule-1 (VCAM-1), lymphocyte function associated antigen-3 (LFA-3) and ICAM-1 in human bronchial epithelial and synovial cells [23]. Taken together, several studies showed an inhibitors effect of macrolides on adhesion molecule expression and thereby, they reduce neutrophil migration into the tissue.
Dapsone is also able to inhibition the expression of cell adhesion molecules [24].

The generation of nitric oxide (NO) is also influenced by some macrolides. NO is an important second messenger in the mechanism of ROS regulation within the inflammatory response. It is produced by the NO synthases (NOS) in the first stages of the inflammatory response and can either enhance vasodilation or influence the migration properties of leukocytes. It has been reported that erythromycin stimulates endothelial NOS (eNOS) production in a protein kinase A-dependent mechanism [25]. This results in inhibition of leukocyte adhesion to endothelial cells and thereby, migration into the tissue. However, there are contradictory reports on suppression of the inducible NOS (iNOS) in pulmonary alveolar macrophages by erythromycin [26]. The results indicate that macrolides can reduce or increase NO synthesis probably depending on the type of NOS with which they interact. Not only macrolides are able to influence the adhesion and migration steps of leukocytes. Clofazimine shows beneficial effects in several skin diseases which might be based on the inhibition of ICAM-1 and HLA-DR [27].

The next important step during the migration process is the attraction of the immune cells by chemokines. A concentration gradient leads the immune cells towards the site of inflammation. The influence of macrolides on neutrophilic chemotaxis is controversial. Several reports showed that long-term treatment with erythromycin reduced the neutrophil chemotactic activity in DPB [28,29]. In different acute lung injury models in mice, erythromycin also showed an inhibiting effect on neutrophil migration [28,30,31]. But macrolides did not show effect on neutrophil chemotaxis in every studies. For example, in vitro models showed that a much higher dose of macrolides than the therapeutic dose was needed to inhibit chemotaxis of isolated peripheral blood neutrophils from healthy volunteers [32]. Some studies even report an enhancing effect of erythromycin on neutrophil migration in vitro. It is difficult to distinguish whether the observed effects are due to reduced chemotaxis or due to reduced production of chemoattractants and/or a decreased expression of adhesion molecules [33,34].

Quinolones also reduce rat macrophage chemotaxis. The effects are concentration-dependent and significant yet they do not provide a promising approach since the effects are not strong enough for clinical application [35]. It has been reported that clofazimine shows inhibitory effects on neutrophil motility ex vivo [36].

The assumption that macrolides inhibit plasma exudation and cell infiltration in vivo is also supported by reports showing the efficacy of these antibacterials in the model of carrageenan-induced paw oedema, which is a standard animal model for the investigation of anti-inflammatory compounds [37]. Pre-treatment with erythromycin had protective effects on inflammatory reactions of the airways in rats after E. coli lipopolysaccharide (LPS)-induction.
These effects seemed to be dependent on neutrophils since there was no protective effect visible in neutropenic rats. This hypothesis is supported by another study which showed that erythromycin and clarithromycin inhibit LPS-induced neutrophil recruitment in the trachea of guinea pigs [39]. In a rat model for lung injury erythromycin and josamycin showed a reduction of NO concentrations in exhaled air and an inhibition of neutrophil accumulation [26].

### 15.2.3. Effects on cellular defense mechanism

After the migration process, the immune cells reach the site of infection where they tackle the invading pathogens. Stimulation of the leukocytes is an essential step in the process at this stage. Neutrophilic granules release a broad range of enzymes, like lysozyme, which directly attack the bacteria. Opsonization of the micro-organisms by immunoglobulins and the complement system enables phagocytosis of the bacteria and the ensuing oxidative burst, which comprises the generation of massive amounts of ROS to destroy the cellular components of the invading pathogens. This process is stimulated by numerous chemokines which further activate the cells and also stimulate other inflammatory mechanisms. The degranulation of neutrophils is directly affected by macrolides in vitro [35]. Additionally, macrolides, with the exception of roxithromycin, stimulate macrophage phagocytosis, chemotaxis and cytocidal activity against the yeast Candida albicans [40]. These host defense promoting effects of macrolides can also be seen in animal models. The administration of erythromycin or roxithromycin (10 mg kg⁻¹) for 28-days promotes pro-inflammatory cytokine production by isolated macrophages and interleukin-2 (IL-2) production by isolated splenocytes in healthy mice. Interestingly, these effects did not occur after a 7-day treatment [41,42]. Furthermore, 14-day roxithromycin treatment in healthy guinea pigs increased the oxidative burst capacity of neutrophils [43]. But not only macrolides are able to boost host defense mechanisms. Quinolones, β-lactams and cephalosporins also promote neutrophil bacterial killing partly by enhancing phagocytosis and oxidative burst capacity in vitro [35]. Nevertheless, granulocyte functions are not affected by quinolones at clinically relevant concentrations [44]. The majority of quinolones, especially ciprofloxacin, influence pro-inflammatory cytokine gene expression like IL-2 and interferon gamma (IFN-γ), by activation of the nuclear factor AP-1, probably accounting for their role as immunomodulators [44]. Studies on the actions of the inflammmagen zymogen A from S. aureus showed that stimulation of THP-1 monocytic cells in vitro by moxifloxacin, a fluoroquinolone, is biphasic [45]. At the start, moxifloxacin enhances the release of hydrogen peroxide and NO, but after 4 hours inhibition of pro-inflammatory cytokines, lipid peroxidation and lysosomal enzyme release occurs. This is an interesting property which could be used for immunomodulatory therapy: The enhancement of the pro-inflammatory processes supports the killing of the invading pathogens while
the anti-inflammatory effects in the second phase enable inflammatory resolution and tissue regeneration. Such biphasic activities have also been reported for azithromycin in studies on healthy humans treated for three consecutive days (500 mg d\(^{-1}\)) [46]. Initially, azithromycin increased the neutrophil degranulating effects but 2.5–24 h after the last dose, the azurophilic granule enzyme activities in the cells decreased while there was a corresponding increase in the serum. *Ex vivo* experiments showed that the oxidative response to particulate stimulus of opsonized zymosan was increased. High concentrations of azithromycin were reported in the serum and in neutrophils. IL-6 and chemokine concentrations in the serum were reduced within the non-pathological range and the oxidative burst capacity was downregulated while the apoptosis of neutrophils was enhanced up to 28 days after the last administered dose. These biphasic effects shown by azithromycin and probably other antibacterials like quinolones, provide a mechanism for an enhanced defense mechanism of the host against invading pathogens and an improved inflammatory resolution to prevent tissue damage through prolonged inflammation.

### 15.2.4. Effects on inflammatory mediators

Several antibacterials show anti-inflammatory effects through inhibition of pro-inflammatory mediators, cytokines and ROS. These antibacterials include macrolides, quinolones and tetracyclines. The anti-inflammatory effects can be made use of due enhance the resolution of inflammation after the successful elimination of the invading pathogens. The most important anti-inflammatory effects of selected antibiotics will be introduced in this chapter.

#### 15.2.4.1. Macrolides

Macrolides have widespread effects on the immune response as partly addressed in the previous chapters. They inhibit the secretion of pro-inflammatory cytokines, ROS and other pro-inflammatory mediators *in vitro* while anti-inflammatory cytokines are also modified in multiple ways [47-49]. The modulation of ROS generation has several immunomodulatory consequences since ROS exert miscellaneous physiological effects. A major consequence of ROS action is cell and tissue damage but ROS are also needed for several physiological cellular signal pathways and regulation processes [50]. Macrolides reduce the production of ROS in neutrophils [51]. As a possible mechanism, it has been proposed that macrolides enhance the membrane-destabilizing effect of bioactive phospholipids (*e.g.* lysophosphatidylcholine, lyso-PAF and platelet-activation factor) and thereby inhibit superoxide production [52]. Erythromycin, clarithromycin and roxithromycin pretreatment of rats with acute carrageenan-induced pleurisy resulted in a reduction of prostaglandin E\(_2\), TNF-\(\alpha\) levels and NO production [33]. These three macrolides also reduced NO, IL-1\(\beta\), IL-6 and TNF-\(\alpha\) plasma levels as well as lung iNOS mRNA level after i.p. injection of LPS into mice.
Interestingly, josamycin did not show these effects [53]. In a similar in vivo experiment with i.p. LPS injections, several modified macrolides were tested. These derivatives do not show antibacterial effects but still are able to reduce cytokine production and neutrophilia in vitro [54]. These findings indicate that the direct antibacterial effects and immunomodulation are partially separable properties and more anti-inflammatory substances can probably be developed based on the structure of macrolides. In general, the anti-inflammatory mechanism of macrolides seems to be rather slow. Studies in a peritonitis model induced by zymosan showed that the anti-inflammatory effects of erythromycin achieve a peak 28 days after (pre-) treatment [55,56]. Roxithromycin also showed a time-dependency, giving the most pronounced reduction of the cytokine secretion (IL-1β, TNF-α) in a mouse endotoxin LPS-induced inflammation, after seven weeks treatment [57]. These long-term effects underlie the use of macrolides in chronic inflammatory diseases. Investigations of the effects of erythromycin and azithromycin in an adjuvant-induced arthritis model in rats showed that anti-inflammatory effects were exerted through a reduction in circulating lysosomal enzyme activities [58]. Furthermore, the prominent transcription factors NF-κB and AP-1, which are involved in a variety of cellular inflammatory mechanisms, are modulated by macrolides [4]. Studies with LPS-primed THP-1 and human peripheral blood leukocytes in vitro have shown that erythromycin, clarithromycin and roxithromycin have an inhibitory effect on the ROS intermediate-induced activation of NF-κB. This effect seems to be dependent on cyclic AMP [59].

15.2.4.2. Quinolones/Fluoroquinolones

Quinolones have direct antibacterial effects through their inhibition of gyrase and topoisomerase IV – enzymes needed for bacterial replication. These enzymes do not exist in eukaryotic cells, but the accumulation of quinolones in mammalian cells can have an effect on the topoisomerase type II enzymes and thereby, exert harmful effects in humans when overdosed [60]. Beside these direct antibacterial effects, fluoroquinolones exhibit several immunomodulatory effects. They influence phagocytic function, T cell response, cytokine production and transepithelial chlorine secretion [35,61]. In vitro, ciprofloxacin and moxifloxacin cause a dose-dependent reduction of IL-4 and IFN-γ generation by T cells [62]. Furthermore, moxifloxacin inhibits IL-1α and TNF-α secretion from LPS-stimulated human monocytes at therapeutic concentrations [63]. Additionally, moxifloxacin suppresses ERK1/2 activation as well as NF-κB translocation in a lung respiratory cell line and in human monocytes [64,65]. Especially moxifloxacin seems to be an agent for the treatment of chronic airway inflammatory diseases since it also inhibits MAPK activation and production of pro-inflammatory cytokines more efficiently than ciprofloxacin or the macrolide azithromycin [66]. Trovafloxacin decreases the production of cytokines (IL-1α, IL-1β, IL-6, IL-10, GM-CSF, TNF-α) in LPS-stimulated monocytes [67]. This immunosuppressive effect is
regulated via the formation of prostaglandin E$_2$ which leads to an increase in intracellular cAMP – a key factor for immunosuppressive effects in monocytes. In LPS-stimulated monocytes, pefloxacin and ciprofloxacin reduce the production of IL-1 at doses below 100 mg L$^{-1}$. Ofloxacin and ciprofloxacin reduce the synthesis of TNF-α at concentrations below 25 mg L$^{-1}$. Grapafloxacin inhibits IL-8 production in TNF-α stimulated cells [68]. In vivo, ciprofloxacin and trovafloxacin were able to rescue mice injected with a lethal dose of LPS by decreasing serum IL-6 and TNF-α levels [69]. Moxifloxacin reduced IL-8 and TNF-α levels and protected immunosuppressed mice from neutrophilic pneumonitis [70]. Taken together, fluoroquinolones reveal anti-inflammatory and immunosuppressive effects indicating a potential beneficial effect in the induction of inflammatory resolution.

15.2.4.3. Fosfomycin

Fosfomycin has a special place among the antibacterials since it is structurally unrelated to any other antibacterial compound. Similar to macrolides, fosfomycin is able to reduce cytokine production by inhibition of NF-κB activation [71,72]. After LPS injection into mice, fosfomycin decreased peak serum levels of IL-1β and TNF-α. Furthermore, local PGE$_2$ and TNF-α concentrations, as well as cyclooxygenase-2 mRNA were reduced in the rat carrageenan air-pouch model. T and B cells were also modulated by fosfomycin and histamine release from basophils was inhibited [35]. Taken together, fosfomycin has immunomodulatory activity which could also be demonstrated in multiple animal models and in clinical trials with severe bronchial asthma patient [35,73].

15.2.4.4. Cyclines/Tetracyclines

Cyclines have direct antibacterial effects through the inhibition of bacterial protein synthesis, as well as immunomodulatory functions via inhibition of different phagocyte functions like cytokine release at clinical relevant concentrations [35]. Beside their antibacterial effects, cyclines have anti-inflammatory and bone resorption-inhibiting effects. Minocycline has been the target of multiple studies in arthritis models and provides a modest improvement in the course of the disease [74]. The anti-inflammatory properties of cyclines are based on the downregulation of the metalloproteinases MMP-2 and MMP-3. Tetracyclines are able to block these enzymes by chelating metal ions and without the cofactor ZN$^{2+}$, the enzyme activity of the metalloproteinase is decreased [75,76]. This effect has been utilized in the treatment of rheumatoid arthritis and in infectious diseases. Numerous beneficial clinical effects of cyclines are based on this protease inhibition. The dimethylamino group at the position C4 of the tetracyclines is essential for the antibacterial activity since removal of this functional group leads to abolition of the antibacterial activity. Only one derivative of this type, CMT-3, retains inhibitory activity against metalloproteinases [77]. Besides
Immunomodulation by antibiotics

blocking metalloproteinases, tetracyclines show further immunomodulatory effects like inhibition of T cell activity and of pro-inflammatory cytokine levels (IL-2, TNF, IL-1) [78,79]. Tetracyclines are also able to act as ROS scavengers thereby, ameliorating the inflammatory state by decreasing tissue damage caused by ROS [24]. Nevertheless, the potential use of tetracyclines in the treatment of chronic inflammatory diseases and bacterial infections has not been fully exploited because of possible side effects and the danger of bacterial resistance.

15.2.4.5. Dapsone

Dapsone has antiprotozoal as well as antibacterial effects and is widely used for the therapy of leprosy – usually in combination with rifampicin [80]. Additionally to direct antimicrobial effects, dapsone shows various immunomodulatory effects like inhibition of ROS generation by neutrophils, competitive inhibition of myeloperoxidase (leading to reduction of pro-inflammatory hypochlorous acid), inhibition of prostaglandin synthesis and inhibition of neutrophil chemotaxis [24]. Some authors have reported inhibitory effects on cytokine production – but mainly at supratherapeutic concentrations [24]. However, recently an inhibitory effect of dapsone was observed on IL-8 mRNA expression and release in human bronchial epithelial cells in vitro at low concentrations comparable to those in the serum of treated patients. In vivo, dapsone also inhibited neutrophil infiltration into the trachea of ferrets treated intratracheally with bacterial LPS [81]. IL-8 plays a major role in skin inflammation and the inhibitory effect of dapsone of IL-8 generation might explain the beneficial effect of dapsone in models of experimentally induced erythema [24]. In clinical practice, dapsone is a first choice drug for the therapy of chronic inflammatory skin diseases with neutrophilic or eosinophilic cell infiltration (e.g. dermatitis herpetiformis, IgA pemphigus, prurigo pigmentosa) [80]. Furthermore, dapsone has been tested for the therapy of rheumatoid arthritis, immune thrombocytopenia and asthma, but no recommendations have been made for these indications [24]. The anti-inflammatory properties of dapsone could be used potentially to reduce the exaggerated inflammation seen during bacterial induced diseases.

15.2.4.6. β-Lactams

One of the first antibiotic substance used in modern medicine was penicillin followed by its derivatives. They act via inhibition of bacterial peptidoglycan synthesis, resulting in bacterial cell lysis. These β-lactam antibiotics are facing bacterial resistance mechanisms mainly as a result of produced β-lactamase. Beside their direct antimicrobial effects, β-lactams inhibit platelet function, undergo dysulfuric reactions as well as causing osmotic diuresis. Furthermore, they inhibit IFN-γ production, but the different β-lactams show variability in potency. Through inhibition of IFN-γ production, β-lactams modulate IL-4 production and IgE synthesis by Th2 T cells [82,83]. IL-1 and TNF-α might also
be influenced by the changes in IFN-γ levels, but other cytokines are not affected by β-lactams in general [84]. Some individual β-lactams have a unique impact on the immune system. Thus, ceftriaxone interacts with the glutamate transporter-1 which is associated with several neurological diseases like stroke, epilepsy and amyotrophic lateral sclerosis. Ceftriaxone has protective effects on neurons and muscles by enhancing the transporter activity [85]. Cefaclor promotes phagocytosis by inducing a pro-inflammatory response of type 1 and increased chemotaxis [86]. Hence, even as far as one of the oldest class of antibacterial agents is concerned, immunomodulatory properties of the compounds are observed.

15.2.5. Effects on inflammatory resolution

Beside their effects on the initiation and progress of the inflammatory response, antibiotics are also able to facilitate the resolution of inflammation through modulation of leucocyte apoptosis. The targeted induction of apoptosis is a promising new approach to the therapy of chronic inflammatory disease and complementary to antibacterial treatment, since it facilitates clearance of damaged tissue [87,88]. Mainly neutrophils are addressed for this purpose since they play a major role in acute inflammation and their apoptosis is a well-regulated process. In the course of host defense, apoptosis is induced in neutrophils by phagocytosis of invading bacteria. Apoptosis promotes specific gene-mediated attenuation of several functional aspects of neutrophils [7]. Apoptotic, but not necrotic, neutrophils are ingested by macrophages [89]. Several macrolides influence the apoptosis of neutrophils and therefore, regulate the clearance mechanisms at the site of inflammation. Some studies showed a pro-apoptotic effect of erythromycin, which is at least partially cAMP-dependent [90]. This mechanism was observed for erythromycin and roxithromycin in isolated human neutrophils and guinea-pig eosinophils stimulated by IL-5 [91,92]. Azithromycin is also able to induce apoptosis in neutrophils without inducing the oxidative burst or pro-inflammatory IL-8 release. Interestingly, this effect was prevented in the presence of S. pneumoniae [93], which might be due to the already high levels of apoptosis in the neutrophils after contact with the bacteria [7]. In a whole blood model, pro-apoptotic properties of erythromycin and azithromycin were shown by flow cytometry [94]. Tilmicosin, a macrolide which is used in veterinary medicine, induced apoptosis in isolated peripheral neutrophils after 2 h incubation [95]. Interestingly, in contrast to azithromycin, the effects of tilmicosin on apoptosis were not affected by the presence of Pasteurella haemolytica. Either the particular type of bacteria influences the apoptosis of neutrophils in diverse ways or the individual macrolides have unique pro-apoptotic effects. A 17-membered tylosine derivative has pro-apoptotic effects in different cell lines [96] while the 16-membered macrolide josamycin has no effect on human neutrophil apoptosis [92]. The modulation of the NF-κB pathway by antibiotics is also controversial. In vitro, inhibition of NF-κB stimulates apoptosis in
granulocytes [87], but an in vivo study showed a more complex mechanism. Thus, during the onset of inflammation, activation of NF-κB leads to pro-inflammatory gene expression while activation during the resolution of inflammation results in anti-inflammatory gene expression and apoptosis [97]. In a study with human volunteers, administration of azithromycin for three days caused initial stimulation of neutrophil degranulation and subsequent prolonged inhibition. Isolated circulating neutrophils showed detectable levels of azithromycin and increased apoptosis for up to 28 days after the last treatment [46]. Taken together, these studies suggest that the effects of macrolides on apoptosis are time-dependent and associated with the status of NF-κB. A more recent study showed that the treatment of human alveolar macrophages in vitro with erythromycin, clarithromycin and azithromycin (14- and 15-membered macrolides) stimulated the phagocytosis of apoptotic neutrophils by macrophages [98]. 16-Membered macrolides like clindamycin were not studied. Additionally, tilmicosin seems to promote macrophage-mediated phagocytosis of neutrophils [95]. Consistent with the other immunomodulating effects of macrolides, the 14- and 15-membered (but not the 16-membered) macrolides are thus, able to increase inflammatory resolution by directly enhancing neutrophil apoptosis and their subsequent phagocytosis by macrophage. Macrolides are not the only antibiotics which influence the mechanisms of apoptosis, however, in many cases apoptosis is delayed or even inhibited. Minocycline inhibits apoptosis in experimental neuroinflammatory disorders [99] and rifampicin inhibits antiCD95-mediated apoptosis in peripheral blood lymphocytes and Jurkat T cells. The mechanism partially involves glucocorticoid receptor activation and the signaling pathway of NF-κB [100,101]. Furthermore, tosufloxacin, as the only quinolone antibiotic, was reported to delay neutrophil apoptosis in vitro. The delay could be reduced by a p38 mitogen-activated protein kinase (MAPK) inhibitor [102]. Taken together, the induction of apoptosis is an essential step in the inflammatory resolution which can be facilitated mainly by macrolides but also other antibacterials.

15.3. CONCLUSION

The modulation of the inflammatory response and the enhancement of host defense is a complex process which can be affected by a broad range of different antibiotics. Macrolides, quinolones and cyclones have the greatest impact. In the early stages of inflammation, macrolides exert inhibitory effects on the adhesion and the subsequent transepithelial migration of leukocytes. Clofazimine might have similar effects in this phase. Leukocytes can be stimulated by these antibacterials as well as by the quinolone moxifloxacin. The stimulation of host defense enhances bacterial killing in addition to the
direct antibacterial effects of the antibiotics and is independent of potential bacterial resistance. The reduction of leukocyte migration and other inflammatory responses by macrolides as well as quinolones leads to a reduction of the inflammation process in vivo. Several antibiotics show ameliorating effects on pro-inflammatory cytokine release which might allow the use of antibiotics in anti-inflammatory approaches as with macrolides in the treatment of DPB [17]. The observation that cyclines are able to inhibit metalloproteinase release and reduce connective tissue breakdown offers several interesting approaches besides their direct antibacterial actions, as in the use of minocycline in periodontal and rheumatic diseases. Not only is the initiation of inflammation influenced by several antibiotics, also the resolution of the inflammatory status is promoted. Thus, macrolides stimulate apoptosis in leukocytes which prevents unwanted tissue destruction subsequent to ongoing inflammation. Their additional effects, like reduction of mucus secretion, enables macrolides to be used in the treatment of respiratory diseases. Beside macrolides, quinolones and cyclines are promising agents for new approaches in the treatment of inflammatory conditions. Sulphasalazine is an example of the use of an antibiotic as anti-inflammatory drug. This transfer from “the classical use” of antibiotics is a promising approach towards the treatment of chronic inflammatory diseases. For the treatment of bacterial infections, the early enhancement of host defense mechanisms is important to reduce the invading pathogens. The time dependent enhancement of apoptosis of the immune cells helps to facilitate the resolution of the inflammatory status to prevent unwanted tissue damage through prolonged inflammation. The major advantage of immunomodulatory antibiotics is that this additional activity is seen even in the presence of bacterial resistance as bacterial killing occurs as a result of enhanced host defense mechanisms and not just through the direct antibacterial properties of the antibiotics.

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Immunomodulation by antibiotics


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PK/PD FOR PREDICTION AND CONTAIN OF ANTIMICROBIAL RESISTANCE

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16.1. INTRODUCTION

In the present scenario, there is an alarming situation of losing treatment due to the increasing microbial resistance against the available antibiotics and the attitude of the pharmaceutical industry, which shows less interest in the production of new antibiotics based on the inadequate return on the investment. Due to the lack of new antibiotics and optimized dosage regimen, rational drug development and usage is becoming a valuable entity for bacterial killing and eradication [1]. The situation is further overwhelmed by the frequent use of antibiotics by the veterinarian, which increase the chances of resistant strains development [2]. Because of these situations, the pharmacokinetic/pharmacodynamic (PK/PD) indices are focused on where the associations between doses, concentrations, and effects (desired and undesired) are defined and quantitated [1].

Bacteria and their susceptibility to an antibacterial drug are usually defined in terms of breakpoints in the advanced microbiology laboratory practices. On the basis of testing technique (agar diffusion, broth dilution and agar dilution), it is either expressed in mg/litre or g/ml in case of concentration, or in mm in case of zone diameter [3]. These are actually biased concentrations which interpret the isolates as susceptible, intermediate or resistant. For setting and adjusting breakpoints, microbiological, pharmacological, clinical and pharmacodynamic approaches are used [4]. However, throughout the world, the established guidelines are used by laboratories for the interpretation of susceptibility tests irrespective of the testing technique. These established guidelines are produced by either European committee on antimicrobial susceptibility testing (EUCAST) or the clinical laboratory standards institute (CLSI). The CLSI guidelines are used in almost all countries outside the European Union, where EUCAST guidelines are followed [5].

Another important parameter for successful therapy is the dosage regimen of a drug, which is usually predicted by evaluating the pharmacodynamics and pharmacokinetic and toxicological data obtained in the preclinical trials. The three main possible approaches are dose titration, and PK/PD modelling and integration. Of these, PK/PD integration is very effective, useful and less expensive compared to the others. PK/PD integration is differentiated from PK/PD modelling on the basis that the former obtains collective data from separate PK and PD studies while the latter is based on the silico modelling of PK and PD data obtained from a single dose of the drug in a single investigation. PK/PD modelling works on the following two mining concepts for drug discovery and development [6]: (i) Has the best compound been selected as a candidate drug? (ii) Has not just an effective but the optimal dosage regimen been established?
It is dire need of the day to develop novel antibiotics, optimize the dosage regimen and guidelines in order to minimize the antimicrobial resistance and make the treatment successful. This review will look at the PK/PD indices, their role in breakpoint selection, optimization of the dosage regimen and how to minimize antimicrobial resistance.

### 16.2. PK/PD FOR PREDICTING THE DEVELOPMENT OF ANTIMICROBIAL RESISTANCE

To avoid the emergence of resistance, the following three main strategies should be used.

The first strategy is based on the concept of changing the exposure time, use of combination therapy, sequential therapy and change in duration of treatment [7]. However, it is not suitable in veterinary medicine, particularly in treating animals for food, but could be used in pets and horses.

The second strategy generally focuses on how to adjust the dose interval in antimicrobial therapies by using the principles of PK/PD to minimize the overuse of antimicrobial agents and individual exposure, thus collectively reducing the chances of bacterial resistance. It is usually pathogen-specific, resulting in safer and more successful therapy with minimization of resistance [8]. In contrast to the first, this strategy could be used in highly valuable animals like horses but could not be used in human medicine because of its huge laboratory setup and knowledge.

The third strategy concentrates on calculation of the correct drug dose in order to overwhelm the amplification of less susceptible mutant bacterial species. This concept must be used in the development of new veterinary drugs. For the prediction of resistance in the bacterial population, a mutant selection window could be used [9]. A semi-mechanistic PK/PD model for the determination of bacterial resistance according to exposure to the antimicrobial agent over time is also useful. A previous study in which colistin methane sulfonate is used against *Pseudomonas aeruginosa* is a good example of this model [10]. Furthermore, this model could be used to study the range of bacterial outcomes, as it analyses pharmacokinetic variability via the population pharmacokinetic model; it is also feasible to study the selection of less susceptible or resistant subpopulations [11]. As it is already practised successfully in veterinary drugs, new drugs should be developed accordingly [12].

The commonly used antimicrobial classes include the β-lactams, aminoglycosides and fluoroquinolones. These agents are grouped on the basis of their involvement in either time-dependent or concentration-dependent killing of micro-organisms (Figure 1). To know the effective outcome for a
particular antimicrobial dosage regimen, the pharmacodynamic targets have been adjusted by combining the animal models, clinical data and in vitro studies [13]. Generally, the following PD parameters are used for predicting the efficacy of antibiotics; however, these PK/PD parameters are now used for reducing the development of resistance in micro-organisms [14].

16.2.1. For time-dependent agents

Previous investigations have reported that the bactericidal activity of the β-lactams is concentration-independent, and the maximum killing of microorganisms occurs at concentrations of three to four times the minimal inhibitory concentration (MIC). Any further increase in drug concentrations has almost no effect on microorganisms. The efficiency of these antibiotics is associated with the percentage of time that the drug concentration remains greater than the MIC (%t > MIC). Previously it has been reported [15] that there is a direct relationship between the efficacy of β-lactam used against *P. Aeruginosa* and %t > MIC. Using the neutropenic mouse model, the researchers reported that for high effectiveness of ticarcillin against *P. aeruginosa*, a %t > MIC value of nearly 100% was required.

One study reported that the area under the curve (AUC)/MIC has a significant role in the emergence of linezolid resistance [16]. They also reported that there are chances of resistance development when the concentration of linezolid is maintained near the MIC value. It is also evident from previous work [17] that attaining concentrations of T>MIC for mutant prevention concentration (MPC) for all dosing intervals is ideal for extreme bactericidal activity and for preventing the emergence of resistance. This is further supported by a study [18] in which T>MIC is selected as a parameter for the evaluation of resistant mutation in *S. aureus*. The authors reported that T>MIC values of 8.11 to 17.14 and 16.21 to 34.28 are the danger zones for induced resistant mutation in groups with *t1/2β* three hours after multiple dosages and in groups with *t1/2β* six hours after multiple dosages.

For the efficacy of time-dependent drugs, the responsible PK/PD parameters are the time and drug concentration above the MIC, which can be measured by the equation given below [19]:

\[
T > \text{MIC} = \ln\left(\frac{D}{V_d \cdot \text{MIC}}\right) \cdot \frac{T_{1/2\beta}}{\ln 2} \cdot \frac{100}{t}.
\]
16.2.2. For concentration-dependent agents

In contrast to β-lactams, the effectiveness of concentration-dependent agents is checked by using $C_{\text{max}}$:MIC, which is a PD parameter. It is documented that as quicker the maximum concentration of a drug in blood or predilection site in the tissue is achieved more rapidly the pathogen will be removed. Generally, for the treatment and inhibition of resistance in gram negative and positive bacteria, a $C_{\text{max}}$:MIC ratio of 8:10 is adopted [20,21]. Actually, this ratio has been accepted for the extreme killing of gram negative pathogens.

In case of $C_{\text{max}}$:MIC failure, the area under the serum concentration-time curve AUC:MIC is used to check the effectiveness of a drug. It effectively describes the efficacy of ketolide, glycopeptide and fluoroquinolone [22]. In particular, the level of risk emergence of fluoroquinolone resistance is best described by this parameter. The reduced *Staphylococcus aureus* susceptibility or resistance to vancomycin is linked with the accessory gene regulator (agr) locus, particularly agr group II in *S. aureus*. Recent studies have reported that for calculating *S. aureus* exposure to vancomycin and the chances of resistance development, AUC/MIC ratio may be used [14].

Previously it has been reported [23] that the ratio of the area under the concentration-time curve from 0 to 24 h to the MIC (AUC$_{0-24}$:MIC) can be used as an important predictor of bacterial resistance ($P < 0.001$). For identification of those factors linked with the development of resistance in bacteria, the authors used the univariate screen and a classification and regression tree. They further explained that at an AUC$_{0-24}$:MIC ratio of less than 100, the chances of bacterial resistance increase significantly during the treatment.

In an *in vivo* *Mycoplasma Gallisepticum* infection model study, the concentrations of danofloxacin were analysed in lung tissues and plasma, followed by the determination of changes in antimicrobial susceptibility and counting of viable cells in air sac and lung tissues. Furthermore, for point mutation identification in *gyrB, gyrA, parE* and *parC*, polymerase chain reaction (PCR) amplification of quinolone resistance-determining regions (QRDRs) and sequencing DNA of selected resistant mutant strains were performed. While analysing the PK profile, it was noted that the danofloxacin concentration was greater in the lung tissue than in the plasma.

The ratios of AUC$_{24}$:MIC for 3 log$_{10}$ (CFU) and 2 log$_{10}$ (CFU) decrease were 97.98 and 31.97 L h kg$^{-1}$, respectively. Replacements of Glu-87→Gly or Ser-83→Arg in *gyrA* and Glu-84→Lys in *parC* were noted in the resistant mutant strains of the dose group 1 and 2.5 mg kg$^{-1}$. MICs of levofloxacin, ofloxacin, gatifloxacin, norfloxacin, enrofloxacin and danofloxacin against the resistant mutant strains with a single mutation in position-83 were higher than in position-87. It was concluded that in the case of *M. gallisepticum*, infection in chicken’s danofloxacin will be effective at a dosage of 5.5 mg kg$^{-1}$ once daily for three days [24].
It has also been reported in several previous works [25-28] that the optimal dosage for all those drugs, the effectiveness of which is linked to $\text{AUC}_{0-24} : \text{MIC}$, can be calculated by the equation given below, which can calculate the dose per day [2]:

$$\text{Dose} = \frac{(\text{AUC}_{24}/\text{MIC}) \cdot \text{MIC} \cdot \text{CL} \cdot \text{fu} \cdot F}{\text{fu} \cdot F},$$

where $\text{AUC}_{24} : \text{MIC}$ is the ratio used for optimal efficacy of routine treatment; MIC is the minimum inhibitory concentration; CL is drug clearance; fu is free fraction and $F$ is bioavailability of drug.

### 16.2.3. Mutant selection window (MSW) and MPC

The MSW concept has been established to define fluoroquinolone resistance. It was postulated to understand how drug exposures beneath the MPC produce circumstances for selecting resistant bacterial strains [22]. In MSW, it is assumed that the lowermost concentration is the lower boundary that applies selective pressure and inhibits bacterial colony formation by 99% (MIC). On the basis of this hypothesis, a possible cause of clinical failure is the concentration of the drug falling within the MSW. One study [18] reported that when $T_{\text{MSW}}$ reaches 36% in groups with $t_{1/2\beta}$ three hours after multiple dosage or above 73% in groups with $t_{1/2\beta}$ six hours after multiple dosages, Cefquinome will limit the resistant mutation. It is the concentration which inhibits the first step mutants [29]. This is a new emerging hypothesis developed to reduce the emergence of resistance [30]. According to this concept, in antibacterial dosing that yields concentrations above the MPC, bacterial resistance will not occur during the dosing interval. It can be achieved for the compound with minute changes in b/w MPC and MIC by increasing the dose and reducing the dosing interval, which will ultimately decrease the time within the MSW [31]. A combined concept of MPC and MSW provides new ways for PK/PD to determine dosing guidelines. In a study conducted in aquaculture, the treatment time was increased, resulting in increased enrofloxacin concentration above MPC at the infection site; when these concentrations were in the MSW, the time was reduced. Therefore, $T > \text{MPC}$ was reported as a substantial parameter for designing the dosage regimen instead of $T > \text{MIC}$ for preventing mutant selection of antimicrobial drugs in aquaculture [32].

For selecting antimicrobial resistance, the ratio between MPC and $\text{AUC}_{0-24}$ is a good predictor [33]. A related study [34] reported that $\text{AUC}_{0-24} : \text{MPC}$ above 25 h limits the attainment of resistance in S. aureus infection. Other studies (in vitro and in vivo) verified that a ratio of $\text{AUC}_{0-24} : \text{MPC} > 22$ h or > 20 h prevents resistance selection in the case of Escherichia coli infection [3,35,36]. It is therefore clear that $T > \text{MPC}$ and reduced time in MSW are suitable parameters for minimizing antibacterial resistance.
Figure 1. Classification of antibiotics on the basis of PK/PD parameters
16.3. PK/PD FOR ESTABLISHMENT OF ANTIMICROBIAL SUSCEPTIBILITY BREAKPOINTS

16.3.1. Definition of antimicrobial susceptibility breakpoints

Antimicrobial susceptibility breakpoint is defined as the antibiotic concentration (mg L\(^{-1}\)) upon which it is decided whether a bacterium is susceptible or resistant. Bacteria are considered susceptible to the antibiotic if the MIC is equal to or less than the susceptibility breakpoint. While defining the clinical, pharmacological and microbiological thresholds, the breakpoints usually generate confusion. To overcome this confusion, it has been suggested [3] that breakpoints should consider three cut-off values, including epidemiological cut-offs, PK/PD cut-offs, and clinical cut-offs.

The epidemiological cut-off (ECV) and wild-type cut-off (\(C_{\text{WT}}\)) are measures of a drug’s MIC distribution that separate bacterial populations into those representatives of a wild-type population and those with acquired or mutational resistance to the drug. A bacterium with a drug MIC that is greater than the ECV is likely to have an acquired form of resistance, whereas one with a drug MIC lower than or equal to the ECV is likely from the wild-type distribution of the bacterium for a particular drug. Principally, this is used to examine whether or not the clinical cut-offs and PK/PD fall below ECV and wild type cut-off and inside the wild-type MIC distribution. If so, then there will be problems in testing and understanding, because certain wild-type bacteria will be susceptible, while others will be either intermediate or resistant. This will further complicate the situation because, as the test results vary on a daily basis, so some bacteria could readily end up in any category. For wild-type distribution of breakpoints, no single solution is yet decided [3].

The PK/PD cut-off (\(C_{\text{PD}}\)) originates from PK/PD modelling, which utilizes knowledge of the antimicrobial PK/PD parameters for the identification of MICs that accurately predict the probability of target achievement for specific bug-drug combinations [35]. In relation to the MIC distribution, it has been suggested [3] that its analysis should be the first step in determining the breakpoint for isolates collected worldwide. The authors further explain that the PK/PD cut-off applied to this collection yields the highest value in this condition by having (i) MIC as an in vitro measurement; (ii) the relevant PD parameter and its magnitude, predicting the in vivo effectiveness; and (iii) human PK and its inter-subject variation and the dosage regimen.

Clinical cut-offs (\(C_{\text{CL}}\)) are based on the collection of isolates obtained during clinical effectiveness studies. They reflect the upper limit of the MIC values linked with a great probability of clinical achievement. The clinical cut-off is often used as a tool of authentication and validation for PK/PD cut-offs. These cut-offs gain weight when they fall below PK/PD cut-offs. In such cases, it is suggested that more PK/PD work should be done to understand the association between PD and its outcomes. Generally, variation in results is
observed when clinical versus PK/PD cutoffs are applied using different dosage regimens in clinical practice. The PK/PD cutoffs are usually applied to specific dosage regimens [3].

16.3.2. Examples of breakpoints development based on PK/PD cutoffs

The first example is the modification of the vancomycin breakpoint for methicillin resistant *S. aureus* (MRSA). Vancomycin was used in the treatment of MRSA infections until 2000 using the trough level < 10 µg mL⁻¹, as it enhances toxicity [36]. Meanwhile, for a better clinical outcome, a trend of higher trough level was introduced by using high doses. This concept was grounded in part on PK/PD data, which suggest that attaining an AUC : MIC ≥ 400 will result in better clinical outcomes [37]. The CLSI in 2006 decreased the clinical breakpoint to ≤ 2 µg mL⁻¹ for MRSA by worrying about the development of heteroresistance, and aggravated clinical outcomes at higher vancomycin MIC values [38]. On the basis of these findings and reports, IDSA guidelines consequently suggest 15–20 µg mL⁻¹ as targeting trough levels in case of severe infections caused by vancomycin-susceptible organisms [35].

The second example is the establishment of penicillin breakpoints for resistant *S. pneumoniae*. Initially, in 1970, the clinical breakpoints for penicillin were recognized in the treatment of meningitis caused by *S. pneumoniae*. Later on, in the 1990s, the ratio of penicillin-resistant *S. pneumonia* increased in the United States; however, higher MIC values of penicillin worked very well in infections other than CNS [39]. Then, in 2008, the CLSI categorized the penicillin clinical breakpoints as non-meningitis (oral), meningitis (intravenous) and non-meningitis (intravenous). It was observed that in the case of meningitis, the pre-2008 cerebrospinal fluid (CSF) breakpoint values [≤ 0.6 µg mL⁻¹ (susceptible) and ≥ 0.12 µg mL⁻¹ (resistant)] did not change, while the serum breakpoints [≥ 8 µg mL⁻¹ (resistant), 4 µg mL⁻¹ (intermediate) and ≤ 2 µg mL⁻¹ (susceptible)] were increased for non-meningitis infections. These clinical breakpoints for non-serum established a new model which promoted the intravenous use of penicillin for pneumococcal pneumonia and similar upper respiratory system infections in case of higher dose recommendation – *i.e.* at least 10 million units/day. Other PK/PD studies recommend that in case of pneumococcal isolates, higher doses of oral amoxicillin with higher penicillin MICs could be used [40].

The third example is the breakpoint of cefazolin for Enterobacteriaceae. In 2011, the CLSI again increased the susceptibility breakpoint for cefazolin from 1 µg mL⁻¹ to 2 µg mL⁻¹, which in 2010 was adjusted from 8 to 1 µg mL⁻¹ against the Enterobacteriaceae. This increase from 1 µg mL⁻¹ to 2 µg mL⁻¹ was done because of a realization that the new breakpoint was too low and would unnecessarily eradicate the use of this drug against *Proteus mirabilis*, *Klebsiella spp* and *E. coli*. However, as the clinical laboratories are never
required to suggest the dosage, it remained unknown to the majority of clinicians. Similarly, The Food and Drug Administration (FDA) is using the old susceptibility breakpoint of $\leq 8 \mu g m L^{-1}$; therefore, several automated antimicrobial susceptibility testing (AST) panels are improved to test a cefazolin MIC of $2 \mu g m L^{-1}$. Hence, laboratories decide themselves how to test the susceptibility of cefazolin [35].

Other examples are breakpoints of $\beta$-lactam, trimethoprim-sulphamethoxazole and sulphafurazole for *Neisseria meningitides*. Using the PK/PD parameters, a study [41] was conducted to develop the break points for *N. meningitides*. The authors used time above the MIC for at least 50% of the dosing interval for all beta-lactams, trimethoprim-sulphamethoxazole, chloramphenicol and sulphafurazole. An AUC:MIC ratio of greater than or equal to 25 and greater than or equal to 125 were used for tetracyclines and macrolides, and fluoroquinolones, respectively. A Monte Carlo simulation of 1000 was prepared (both serum and CSF) at MIC values of 0.03–64 mg L$^{-1}$ for each antimicrobial agent. Furthermore, the authors proposed that the PK/PD breakpoint would be the MIC, where the calculated target attainment would be greater than or equal to 95%. Based on these assumptions, the suggested PK/PD breakpoints were $0.125 \text{ mg L}^{-1}$, $0.25 \text{ mg L}^{-1}$, $0.5 \text{ mg L}^{-1}$, $1 \text{ mg L}^{-1}$, $2 \text{ mg L}^{-1}$ and $4 \text{ mg L}^{-1}$ for azithromycin; doxycycline; cefotaxime, ciprofloxacin and levofloxacin; penicillin G, meropenem, rifampicin, tetracycline and minocycline; chloramphenicol and sulphafurazole and ampicillin, ceftriaxone; and trimethoprim-sulphamethoxazole. For CSF, the proposed PK/PD breakpoints were $0.06 \text{ mg L}^{-1}$, $0.125 \text{ mg L}^{-1}$, $0.25 \text{ mg L}^{-1}$, $0.5 \text{ mg L}^{-1}$ and $1 \text{ mg L}^{-1}$ for penicillin and cefotaxime; rifampicin; ceftriaxone, meropenem and trimethoprim-sulphamethoxazole; and ampicillin and chloramphenicol, respectively.

### 16.3.3. Comparison of breakpoints in CLSI and EUCAST

CLSI and EUCAST have recommended the establishment of breakpoints by PK/PD cut-offs. Over the last few decades, in various bacterial clinical pathogens, the emergence and spread of antibiotic resistance has been noted. One of the most popular guidelines used throughout the world is CLSI. Their cut-offs for various antibacterials depend on distributions of MIC, PK/PD properties and resistance mechanisms. Later on, in 1997, for the formation of the EUCAST, various national agencies in Europe harmonized for the selection of antibiotic interpretive breakpoints. Now most of the European countries have converted to EUCAST guidelines from CLSI. The clinical breakpoints of EUCAST are based on PK/PD properties and epidemiological MIC cut-offs [42]. The differences in their points of view on the breakpoints of antimicrobials are summarized in the following Tables 1 and 2.
## Table 1. EUCAST and CLSI comparison of breakpoints and media selection for different microorganisms

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameter</th>
<th>CLSI</th>
<th>EUCAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Setting for Breakpoint</td>
<td>+ Microbiological</td>
<td>+ PK/PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ PK/PD</td>
<td>+ Microbiological</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+ Clinical Outcomes</td>
<td>+ Clinical Outcomes</td>
</tr>
<tr>
<td></td>
<td>Media selection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Streptococcus spp.</td>
<td>MH+5 % sheep(disk) MH+2.25–5 % LH</td>
<td>Mueller-Hinton F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(BMD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pasteurella multocida (spp.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Campylobacter jejuni/coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Haemophilus influenza (+para)</td>
<td>Heamophilus test medium</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Listeria monocytogenes</td>
<td>MH+2.25–5 % LH (BMD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corynebacterium spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M. catarrhalis</td>
<td>MHB &amp; MHA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N. gonorrhoeae</td>
<td>GC agar + Suppl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Helicobacter pylori</td>
<td>MH + 5 % sheep aged (disk)</td>
<td>(MIC method)</td>
</tr>
<tr>
<td></td>
<td>N. meningitidis</td>
<td>MH+5 % sheep(disk) MH+2.25–5 % LH</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(BMD)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anaerobes</td>
<td>Brucella + Haemin + Vit K [agar Dilution, add LHB for BMD]</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. On the basis of EUCAST 2015 and CLSI 2015 guidelines, differences in the susceptibilities of E. coli, P. aeruginosa and S. aureus to various antibiotics: concordance and kappa statistics

<table>
<thead>
<tr>
<th>S. No</th>
<th>Micro-organism</th>
<th>Antibiotic</th>
<th>CLSI (%); n = 532</th>
<th>EUCAST (%); n = 532</th>
<th>Concordance (%)</th>
<th>Kappa, $\kappa$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>1</td>
<td>P. aeruginosa</td>
<td>Amikacin</td>
<td>79.5</td>
<td>3.0</td>
<td>17.5</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceftazidime</td>
<td>70.9</td>
<td>4.7</td>
<td>24.4</td>
<td>70.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cefepime</td>
<td>72.6</td>
<td>22.7</td>
<td>27.4</td>
<td>72.6</td>
</tr>
<tr>
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<td></td>
<td>Ciprofloxacin</td>
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<td>5.3</td>
<td>22.9</td>
<td>66.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin</td>
<td>72.6</td>
<td>6.2</td>
<td>21.2</td>
<td>72.6</td>
</tr>
<tr>
<td>2</td>
<td>S. aureus</td>
<td>Penicillin</td>
<td>10.6</td>
<td>0</td>
<td>89.4</td>
<td>10.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Levofloxacin</td>
<td>90.8</td>
<td>0.3</td>
<td>8.9</td>
<td>90.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Clindamycin</td>
<td>98</td>
<td>1.7</td>
<td>0</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamycin</td>
<td>96.3</td>
<td>0.5</td>
<td>3.2</td>
<td>91.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erythromycin</td>
<td>85.6</td>
<td>13.9</td>
<td>11</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>E. coli</td>
<td>Amikacin</td>
<td>99.3</td>
<td>0.3</td>
<td>0.4</td>
<td>90.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gentamicin</td>
<td>78.5</td>
<td>0.2</td>
<td>21.3</td>
<td>78.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cefepime</td>
<td>80.5</td>
<td>1.0</td>
<td>9.5</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ceftazidime</td>
<td>76.3</td>
<td>0.9</td>
<td>22.8</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ampicillin</td>
<td>13.8</td>
<td>0.5</td>
<td>85.7</td>
<td>13.8</td>
</tr>
</tbody>
</table>
16.4. PK/PD FOR OPTIMIZATION OF DOSE REGIMEN

The level of drug concentration in blood and its long-term maintenance close to a therapeutic value is very important for the treatment of many diseases. A dosage regimen which reduces under- and over-exposure to the target concentration increases the potency and safety, resulting in the successful recovery of the patient [43].

On the basis of the increased emergence of resistant organisms and their poor response to the existing antimicrobial agents and unpredictable pharmacokinetic changes in some patients, treatment of infectious diseases is becoming increasingly challenging. Therefore, there is an urgent need for novel strategies for dose optimization to reuse older and forgotten antibacterials and increase the efficacy of the existing one [44]. As has already been reported, bacterial exposure to sub-optimal concentration of an antimicrobial agent is an important factor in the emergence of resistance [45]; therefore, some strategies based on PK/PD parameters regarding the dosing regimen have been established (Figure 2) to help in minimizing the selection of antibiotic resistance [2].

![Figure 2. Different approaches used for dose optimization on basis of PK/PD parameters](image-url)
A study conducted on PK/PD integration for optimization of the cefquinome dose against *S. aureus* [2] reported that for a time-dependent drug, dose determination is better than AUC:MIC and analysis of the number of viable bacteria after 24 h. This statement is supported by another study [46], which documented that PK/PD modelling is another important method for calculating the effectiveness of antimicrobials and predicted gentamicin as a successful treatment against *P. multocida*, with optimum daily dosage in buffalo calves being 2–2.5 mg kg\(^{-1}\) (MIC \(90 \leq 1.0 \, \mu\text{g mL}^{-1}\)). However, in serious and difficult clinical cases caused by pathogens of MIC \(90 \leq 4.0 \, \mu\text{g mL}^{-1}\), the authors recommended a dose of 7.5 mg kg\(^{-1}\). They further suggested that in case of *P. multocida*, a low MPC of gentamicin means that there is a low selection pressure for amplification and resistance emergence in the subpopulation during the treatment.

A study conducted into the concept that PK/PD can be used in everyday clinical practice [47] reported that in antimicrobial therapy some obstacles have to be overcome. These obstacles include rapid and accurate isolation of the pathogen, which is generally used to minimize the time needed for measurement of MIC and plasma concentrations to know the individual patient’s PK system, and final agreement on the PK/PD markers, including the relative breakpoints. However, these problems could be solved by using the population PK model, which provides many benefits and brings pharmaceutical care to a new level. Similarly, a study was conducted in veterinary medicine for optimization of the dosage regimen using PK/PD models [6]. The authors reported that instead of dose titration studies, the parameters obtained from PK/PD modelling may be used as an alternative for rational dosage regimen selection in clinical trials. PK/PD modelling is not in practice in the field of veterinary medicine because of limited studies and appreciation of PK/PD principles in the veterinary scientific community, its limited understanding and its absence from the guideline issued by the regulatory bodies. The factors which influence the design of a safe and effective dosage regimen are shown in the figure below (Figure 3).
16.5. CONCLUSION

PK/PD modelling is a technique that critically analyses quantitative data and establishes a relationship among dose, exposure and response to antibiotics. From this review, it is concluded that PK/PD indices/modelling have introduced and opened new doors for minimizing bacterial resistance and created new hope for clinicians in the sense of establishing an effective dosage regimen. On the basis of this set of tools, a mechanism on the basis of PK/PD modelling of an optimal dosage regimen can be developed for novel and established antibiotics with high efficacy and minimum chance of bacterial resistance development.
REFERENCES