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Alternatives to overcoming bacterial resistances: *State-of-the-art*

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ABSTRACT

Worldwide, bacterial resistance to chemical antibiotics has reached such a high level that endangers public health. Presently, the adoption of alternative strategies that promote the elimination of resistant microbial strains from the environment is of utmost importance. This review discusses and analyses several (potential) alternative strategies to current chemical antibiotics. Bacteriophage (or phage) therapy, although not new, makes use of strictly lytic phage particles as an alternative, or a complement, in the antimicrobial treatment of bacterial infections. It is being rediscovered as a safe method, because these biological entities devoid of any metabolic machinery do not possess any affinity whatsoever to eukaryotic cells. Lysin therapy is also recognized as an innovative antimicrobial therapeutic option, since the topical administration of preparations containing purified recombinant lysins with amounts in the order of nanograms, in infections caused by Gram-positive bacteria, demonstrated a high therapeutic potential by causing immediate lysis of the target bacterial cells. Additionally, this therapy exhibits the potential to act synergistically when combined with certain chemical antibiotics already available on the market. Another potential alternative antimicrobial therapy is based on the use of antimicrobial peptides (AMPs), amphiphilic polypeptides that cause disruption of the bacterial membrane and can be used in the treatment of bacterial, fungal and viral infections, in the prevention of biofilm formation, and as antitumoral agents. Interestingly, bacteriocins are a common strategy of bacterial defense against other bacterial agents, eliminating the potential opponents of the former and increasing the number of available nutrients in the environment for their own growth. They can be applied in the food industry as biopreservatives and as probiotics, and also in fighting multi-resistant bacterial strains. The use of antibacterial antibodies promises to be extremely safe and effective. Additionally, vaccination emerges as one of the most promising preventive strategies. All these will be tackled in detail in this review paper.
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1. Introduction

Worldwide, bacterial resistance to antimicrobial therapy has increased dramatically over the past few years, reaching a "new pre-antibiotic era", where society is put in danger. Currently, according to the World Alliance against Antibiotic Resistance (WAAR), antibiotics may completely lose their effectiveness over the next five years due to a combination of both self-medication and irrational prescription and use of these therapeutic agents, which has led to the development of multi-resistant bacterial strains, and in fact, some of them are resistant to all available antibiotics. Therefore, the need to develop feasible alternatives to antibiotics becomes more and more of utmost importance, so as to protect and promote global public health (Goossens et al., 2005; Carlet et al., 2011, 2012b; Carlet and Mainardi, 2012; Escobar-Paramo et al., 2012; Oldfield and Feng, 2014; WHO, 2015).

The European Centre for Disease Prevention and Control (ECDC) reported that, each year, 25000 people die from infections caused by multi-resistant bacteria and also added that these microorganisms costs about 1.5 billion euros in extra healthcare services and productivity losses per year to Europe (Carlet and Mainardi, 2012). Additionally, as published in a report by the World Health Organization (Leung et al., 2011), in the USA alone such costs represents ca. 35 billion USD per year. It was found that, certain bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* (which are both pathogenic and commensal bacteria in humans or animals), that in an initial phase were susceptible to conventional antibiotics, started to acquire resistance to the antimicrobial treatment, including to the third-generation cephalosporins (Carlet et al., 2011; Carlet and Mainardi, 2012).

The "globalization of resistance" to antibiotics occurred, for example, with the spread of New Delhi Metallo-beta-lactamase-1 (NDM-1), an enzyme produced by *Klebsiella pneumoniae* or *Escherichia coli*, which can make them resistant to β-lactam antibiotics. This enzyme was first discovered in India, and later it was disseminated to Pakistan, USA, Canada, Japan and the United Kingdom (Yong et al., 2009; Walsh, 2011; Charan et al., 2012; Tsang et al., 2012; Ojala et al., 2013). Another example of "globalization of resistance" to antibiotics is the appearance, back in 2009, of KPC (*Klebsiella pneumoniae* carbapemenase), an enzyme responsible for the degradation of carbapenem antibiotics (Nordmann et al., 2011). The panorama is more alarming when bacterial resistance linked to bacterial virulence factors, leading to an outbreak as what took place with the haemolytic-uremic syndrome associated with the *Escherichia coli* serotype O104:H4, which occurred in Europe in May 2011, affecting more than 3400 people and killing ca. 50 patients. This bacterial strain was found in salads, causing renal failure, thrombocytopenia and haemolytic anemia (Buchholz et al., 2011).

Bacteria can resist to antibiotics via different resistance mechanisms, viz. (i) reduction in bacterial uptake of the antibiotic; (ii) production of hydrolytic enzymes, such as β-lactamases, that inactivate the antimicrobial drug; (iii) modification of the antimicrobial drug receptor; (iv) reduction of the antimicrobial drug concentration in the intracellular environment by the efflux pumps present in bacterial membranes; (v) modification of the enzymatic pathway, leading to a decrease of the bacterial susceptibility to the antibiotic; and (vi) loss of intracellular enzymes used in the activation of the prodrug (Łęski and Tomasz, 2005; Piddock, 2006; Rouveix, 2007; Stavri et al., 2007; Rang et al., 2012; Romanelli et al., 2010; Wardal et al., 2010; Martínez-Júlvez et al., 2012; Ojala et al., 2013).

Over the last decades, it has been observed an increased use of antibiotics, in large part caused by the increasing numbers of people needing healthcare, as a result of an ageing population and the consequent increase of chronic diseases and healthcare-associated infections (HAI), also known as nosocomial infections, which represents a worldwide public health problem (Pina et al., 2010a,b; Fair and Yitzhak, 2014).

Self-medication provides another "weight" factor that contributes to a widening of this problem and leads to an overconsumption of antibiotics. Drugs are incorrectly used by the population, and are frequently used to treat common colds and/or respiratory tract infections, mainly caused by viruses (Campos et al., 2007; Mainous et al., 2009). Furthermore, the overuse of

antibiotics in farm animals, either for therapeutic or prophylactic purposes or even as growth promoters, makes the situation even more dramatic because it induces the selection of pathogenic and commensal microorganisms that are resistant to pharmacological antibiotic molecules (Aarestrup et al., 2000; Donabedian et al., 2003; Wegener, 2003; Jacoby, 2005; Johnsen et al., 2005; Katsunuma et al., 2008).

The discovery of penicillin by Alexander Flemming in 1928 changed radically the history of medicine, treating infections that until then there was no way they could be treated. This new agent started to be utilized in large scale, greatly reducing the rates of morbidity and mortality associated with infectious diseases (Aminov, 2010). Despite the great initial success, soon there was news of microorganisms resistant to the new drug class (Abraham and Chain, 1988). The use of the new class of drugs (antibiotics) was disseminated and, with it, the patterns of resistance. Data from the European Union show that ca. 25,000 persons die annually victims of infections by multiresistant microorganisms. In the United States of America, this number reaches an astounding 63,000 deaths per year (Aminov, 2010). The causes for the appearance and dissemination of bacterial resistance are complex and multifactorial. The major causes are: (i) *Overuse*. Recent data show that at least 58% of the children with diagnostic of influenza received antibiotics. The unnecessary use of antibiotics selects resistant specimens of normal microbiota, contributing for the dissemination of resistance genes (Nitsch-Osuch et al., 2016). The lack of information or of diagnosis criteria have contributed largely for the excessive use of these pharmaceuticals; (ii) *Inadequate prescriptions*. Beyond exaggerate use, the number of inadequate prescriptions is also surprising. Studies show errors in the choice of drug and treatment time of about 30–50% of the employment of antibiotics (Ventola, 2015). Subtherapeutic doses of antibiotics induce phenotypic changes in bacteria contributing to the emergence of bacterial resistance (Viswanathan, 2014); (iii) *Use in agriculture and livestock*. In 2011, in the United States of America, it is estimated that the use, only as growth promoters in livestock, was more than 13000 tons of antibiotics. Meat production in the same year was over 42 million tons, which would give an average of almost 320 mg of antibiotic per kilogram of meat produced in the United States alone (Aarestrup, 2015). The use of antibiotics in meat production has been regarded as largely responsible for the increase in bacterial resistance indicators (Boerlin and Reid-Smith, 2008). By ingesting large amounts of antibiotics, there will be death of susceptible bacteria, with consequent prevalence of resistant organisms. By eating the meat of those animals, the human being acquires these microorganisms, pathogenic or not, but which carry resistance genes, exposing the humans to infections untreatable with antibiotics (Ventola, 2015). Bacterial resistance was reported by the WHO (World Health Organization) and the CDC (Centers for Disease Control and Prevention) as a global threat for the 21st century. The rise of enzymes that degrade carbapenems (KPC-2 – *Klebsiella pneumoniae* carbapenemase-2) and beta-lactam antibiotics (NDM-1 – New Delhi metallo-β-lactamase-1) has placed even further concern in the scientific community worldwide, due to the lack of therapeutic options for the treatment of infections caused by these microorganisms (Liu et al., 2016). For such treatment, the only available options are tigecycline and colistin, used alone or in combination (Falagas et al., 2011).

Finally, yet importantly, resistance to antibiotics has reached such a high level that endangers the human race itself, and with the current crisis both in the European Union and in South-America, the funds available to research and healthcare will be reduced so it can be expected a faster and easier spreading of these resistant bacterial strains in the community and hospitals (Meir et al., 2011). Hence, it becomes urgent to discover new antimicrobial agents with different mechanisms of action from those currently available on the market, as well as new ways to relate with infectious diseases (viz., vaccines, probiotics, among others), and new therapies for the treatment and prevention of infections. It can be an arduous, time-consuming and extremely expensive task, because every year only 0.01% of the new molecules tested (5 in 260,000–530,000) exhibit antimicrobial activity, added to the fact that they have huge production costs, high levels of toxicity and extremely complex synthesis (Fernebro, 2011; Ojala et al., 2013).

It is imperative that new actions are carried out both at the community level and hospitals, in order to change the way in which bacterial infections are diagnosed and treated. It is also crucial to adopt alternative strategies that promote the elimination of these resistant bacterial strains from the environment, namely: (i) phage therapy; (ii) lysin therapy; (iii) bacteriocins; among other potential strategies (Hanlon, 2007; Almeida et al., 2009; Housby and Mann, 2009; Theuretzbacher, 2009; Abedon et al., 2011; Fernebro, 2011; Freire-Moran et al., 2011; Wijagkanalan et al., 2011; Carlet et al., 2012a; Escobar-Paramo et al., 2012; Parracho et al., 2012). Hence, the major goal of this review paper was focused on the research, compilation and analysis of the antimicrobial strategies outlined above, which present themselves as potential and viable alternatives to conventional antimicrobial chemotherapy.

2. Bacteriophages and phage therapy

Bacteriophages, or phages, are viruses that only infect bacterial cells. They are biological entities known for over a century. Phage particles represent the most abundant biological entities on the planet, and total phage abundance in the biosphere has been estimated at 10^{30}, or more (Chibani-Chennoufi et al., 2004). However, only now a special interest on phages has been re-discovered, as a potential alternative or complement to current antimicrobial chemotherapy due to their highly specific and unique properties to fight bacterial strains resistant to conventional antimicrobial drugs (Hermoso et al., 2007; Housby and Mann, 2009; Hagens and Loessner, 2010; Summers, 2012). Phages are biological entities completely devoid of any metabolic machinery, and thus are obligate intracellular parasites that require a bacterium to replicate themselves, through their genetic material, by taking over the biochemical machinery of the bacterial cells (Skurnik and Strauch, 2006; Hermoso et al., 2007; Hyman and Abedon, 2010).

Most phages discovered until the present day are specialized in interacting with bacteria that express specific receptors and, if the bacteria does not show at the surface a specific receptor for a particular bacteriophage, then the phage becomes naturally (and highly) specific for a given bacterial host. It is estimated that for every bacterial cell, there are ten different bacteriophages, some of which are highly specific for their host – meaning that they recognize only one type of receptor (monophage), while others have a broader host range and recognize more than one type of receptor (polyphage) (Skurnik and Strauch, 2006; Hyman and Abedon, 2010; Chan and Abedon, 2012).

Phage therapy has been applied over the past few decades to the treatment of bacterial infections, in countries such as The Republic of Georgia (Eliava Institute and The Center for Phage Therapy, in Tbilisi) and Poland (Institute of Immunology and Experimental Therapy, in Warsaw), where research and development centres were built specifically for bacteriophages aiming at developing phage therapy. The studies conducted in these research centres produced remarkable clinical results. However, and despite the immense potential of bacteriophages for eradicating infections caused by bacterial-resistant strains, up to now only a few clinical trials have been performed in human beings and are accepted by public health authorities, such as Food and Drug Administra-

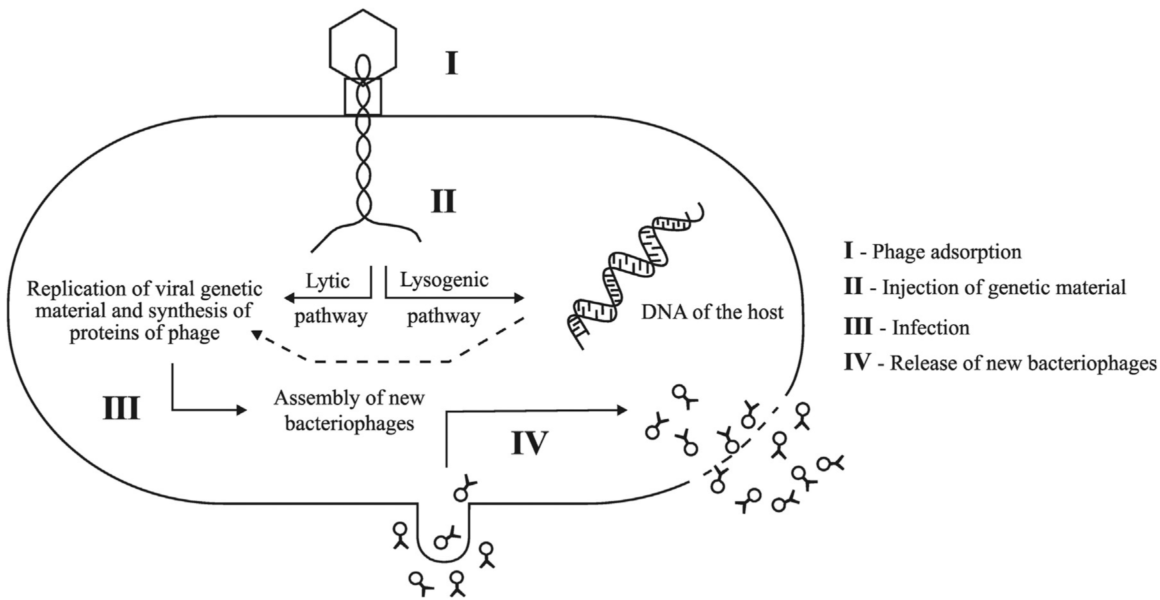

Fig. 1. Schematic diagram of the lytic and lysogenic infectious cycles of a bacteriophage.
Source: adapted from Maura and Debarbieux (2011).

tion (FDA) and European Medicines Agency (EMA) (O'Flaherty et al., 2009; Maura and Debarbieux, 2011).

Concerning their morphology and classification, phages can be divided into six major groups. However, globally speaking, they all exhibit a well-defined structure, usually with an icosahedral capsid involving the genetic material. Within the capsid's core, they may contain single stranded (ssDNA) or double stranded (dsDNA) DNA, or single stranded (ssRNA) or double stranded (dsRNA) RNA, as shown in Table 1 (adapted from Ackermann (2007) and Hanlon (2007)).

Since the 1960s more than 5100 phages have been studied, allowing to conclude that ca. 96% of them possess tails and belong to *Myoviridae, Siphoviridae* and *Podoviridae* families (Dabrowska et al., 2005; Ackermann, 2007; Hanlon, 2007; Wittebole et al., 2013).

2.1. Phage infection process: *lysogenic pathway* vs. *lytic pathway*

The process of bacterial infection by its predator (*i.e.*, the bacteriophage) (see Fig. 1) begins when bacteriophage adsorbs onto the surface of the bacterial cell. It is a complex process, consisting of three phases. Initially, there is a contact between phage and bacterium by diffusion and Brownian motions. Subsequently, the phage particle establishes a reversible, nonspecific binding via electrostatic forces, followed by an irreversible binding between the capsid protein of phage and bacterial surface receptor which, depending on the type of phage in question, may be: (i) a glycoprotein, (ii) a lipopolysaccharide, (iii) an aminoacid, (iv) a teichoic acid, or (v) *pili* (Skurnik and Strauch, 2006; Maura and Debarbieux, 2011; Wittebole et al., 2013). After binding of the phage to the bacterial cell surface, a portion of the phage structure (or the phage itself) penetrates into the host, followed by a complete release of the genetic material into the host's intracellular environment.

Depending on the type of phage, either a lytic or a lysogenic response can take place following bacterial infection. Considering the lytic response, caused by strictly lytic phages also commonly called virulent phages, the bacterial host's metabolism is assaulted and re-targeted to the production of new phage particles, by replicating the genetic material of the virus into the cytoplasm, leading to the synthesis of more lytic phage particles in cycles of 30 min which, aided by produced holins and lysins, subsequently causes lysis of the bacterium. It is also important to refer that the genetic material of the phage is methylated by a DNA adenine methylase, which introduces a methyl group into the carbon 5 of the pyrimidine ring of a cytosine, thereby protecting the exogenous DNA from the destructive action of restriction endonucleases found in the cytoplasm of bacterial cells. Initially, the RNA polymerase is drawn to the site due to the presence of a promoter sequence in the phage genome, and the transcription process leads to the production of viral mRNA which is then translated, leading to the synthesis of viral proteins; this not only inhibits the transcription process of the host's own genome, but both stimulates the replication of the exogenous genetic material and allows the taking over and control of the bacterial cellular machinery, and stimulates the late replication of some genes that encode the capsid proteins and enzymes (holins and lysins) that cause the lysis of the cell wall, which are essential for the release of new virions. This is the type of phage particles sought and used in phage therapy (Weinbauer, 2004; O'Flaherty et al., 2009; Maura and Debarbieux, 2011; Wittebole et al., 2013).

In case of a lysogenic response caused by temperate phages, the phage reproduction happens on a later stage, because the viral genetic material is integrated into the genome of the bacterium. In this situation, the phage is replicated without lysing the host and the bacterium becomes immune to attacks of other phages of the same strain, becoming a lysogenic (and usually more virulent) bacterium (Weinbauer, 2004; Hanlon, 2007; O'Flaherty et al., 2009; Maura and Debarbieux, 2011; Wittebole et al., 2013). This bacterium is characterized by having a prophage, an inactive phage that is integrated in its genome and remains in a latent state for several bacterial cell divisions. The prophage is activated following *stress* processes or cellular damage of the host, inducing its replication via a lytic pathway, after it exits the bacterial genome. There is then the synthesis and release of new virion particles via extrusion, without occurring the disruption of the bacterial cell membrane (Weinbauer, 2004; Hanlon, 2007; Lu and Collins, 2009; Maura and Debarbieux, 2011; Wittebole et al., 2013).

There is currently a special interest in this type of (lysogenic) phages because, despite these phages can transfer genes containing resistance to antibiotics to another non-resistant bacterial strains, this might be an innovative process that can be exploited from

Table 1
Classification of bacteriophages according to their morphology and type of genetic material.

Structure	Family	Nucleic acid	Morphology	Examples
	Myoviridae	Linear dsDNA	Nonenveloped; Contractile tail	T4
	Siphoviridae	Linear dsDNA	Nonenveloped; Long and noncontractile tail	λ
	Podoviridae	Linear dsDNA	Nonenveloped; Short and noncontractile tail	T7
	Microviridae	Circular ssDNA	Nonenveloped; Isometric	φX174
	Corticoviridae	Circular dsDNA	Nonenveloped; Isometric	PM2
	Tectiviridae	Linear dsDNA	Nonenveloped; Isometric	PRD1
	Leviviridae	Linear ssRNA	Nonenveloped; Isometric	MS2
	Cystoviridae	Segmented dsRNA	Enveloped; Spherical	φ6
	Inoviridae	Circular ssDNA	Nonenveloped; Filamentous	fd
	Plasmaviridae	Circular dsDNA	Enveloped; Pleomorphic	MVL2

Source: adapted from Ackermann (2007).

a therapeutic perspective. Such process makes use of genetically modified lysogenic phages to insert, via lysogenization, specific genes into the genome of resistant bacterium, increasing its susceptibility to a given class of antibiotics (Lu and Collins, 2009; Edgar et al., 2012; Wittebole et al., 2013).

It is essential to note that during the process of phage infection, the phage ensures that only its viral DNA is replicated, avoiding, in this way, the interference of other phages in the process. Finally, in both cycles, after the synthesis of viral proteins and enzymes responsible for capsid formation and the packaging of genetic material, there is the assembly and formation of new virions. These disrupt the cytoplasmic membrane, aided by holins, and then the peptidoglycan layer, aided by lysins, leading to the lysis of the bacterium (lytic process), and thereby expelling the newly formed virions to the extracellular environment (Ackermann, 2007; Maura and Debarbieux, 2011; Wittebole et al., 2013).

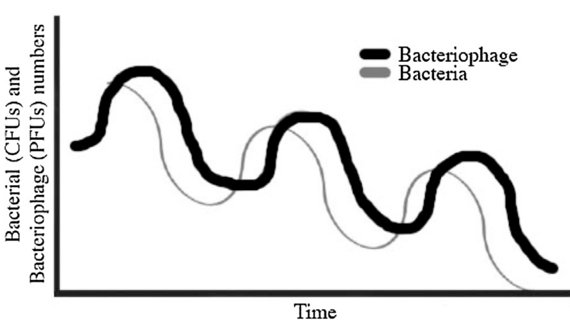

Fig. 2. Reduction in both bacterial numbers (CFU – Colony Forming Units) and phage particle numbers (PFU – Plaque Forming Units) during the process of lytic infection by multiple bacteriophages.
Source: adapted from Parracho et al. (2012).

2.2. Phage therapy and its pre-requisites

Phage therapy is one of the potential alternative strategies to antibiotherapy, and consists in using strictly lytic bacteriophages as an alternative or complement in the treatment of bacterial infections (Escobar-Paramo et al., 2012; Chan and Abedon, 2012). Despite their status as viruses, bacteriophages are strictly specific to certain prokaryotic cells, which include all pathogenic bacteria known until now. The phage therapy has been proven to be safe and free from adverse side effects, and it is worthy to remember that these biological entities are totally devoid of any metabolic machinery on their own and that they have no affinity whatsoever to eukaryotic cells (Chan and Abedon, 2012).

Strictly lytic phages are the natural predators of bacteria and, when in contact with them, they invade specific bacterial strains and induce a lytic infection process associated with a metabolic disruption and cell lysis, reducing the number of bacterial cells present in the infected human host to a number that does not represent a danger to the organism, as illustrated in Fig. 2 (adapted from Kutter et al. (2010) and Escobar-Paramo et al. (2012)).

The exclusive use of monophages as therapeutic agents may not be optimal, because due to the fact that they have a reduced spectrum of action, they may limit the potential of phage therapy in the treatment of bacterial infections, particularly when the bacterial strain that causes the infection is not known. Thus, the spectrum of action can be extended through the use of a phage cocktail, which is a mixture of strictly lytic bacteriophages infecting a wider range of hosts (*i.e.*, several bacterial strains of the same species or different species simultaneously) (Chan and Abedon, 2012).

There is a set of pre-requisites for phage therapy that aims to avoid any failure of this therapeutic approach. Such requirements stipulate that: (i) only phages that have an exclusively lytic

activity may be applied in phage therapy; (ii) before applying the antimicrobial therapy with a particular phage, it is absolutely necessary to know the biology and the host range that affects it; (iii) phage preparations must be absolutely free of bacteria and its components, such as proteins (and, specifically, bacterial endolysins), DNA, organelles, among others; (iv) the bacterial receptor where the phage can act is to be well-known, since a mutation that causes a change in the receptor leads to ineffective phage therapy; (v) the phage preparation must be tested in animal models, to ensure therapeutic efficacy, because it may exhibit a different activity *in vivo* (Levin and Bull, 2004; Skurnik and Strauch, 2006; Hermoso et al., 2007; Viertel et al., 2014).

2.3. Advantages and disadvantages of phage therapy when compared to chemical antibiotherapy

The advantages of phages in relation to conventional antibiotics are several, namely: (i) bacteriophage particles are highly specific to a particular bacterial species or strain, depending on the kind of receptor that recognizes them; (ii) they are the natural predators of bacteria; (iii) the community is constantly exposed to these biological (although metabolically inert) entities; (iv) they are ubiquitous in the environment and one of the most abundant entities on the planet; (v) they are easily isolated; (vi) they exhibit high tissue permeability and, concomitantly, a good skin penetration, being able to reach deeper layers of the skin where chronic (infected) wounds may lie; (vii) they can be applied to eradicate pathogenic bacteria without affecting the commensal flora, hence preventing secondary infections; (viii) they do not affect eukaryotic cells, as they have no affinity whatsoever for them; (ix) they present an exponential growth and replicate inside the bacteria and, therefore, accumulate in extremely high concentrations at the site of infection, as long as its bacterial host still exists; (x) successive administrations of phage preparations become fully unnecessary, since while the target bacterium is present phage particles will constantly replicate until the bacterial concentration drops to a value that does not present a danger to the organism in question, being subsequently (and naturally) eliminated by the body; (xi) they possess a high ability to penetrate bacterial biofilms; (xii) they can mutate and overcome bacterial resistance; (xiii) the isolation procedure of new bacteriophages is fairly simple and economical, unlike the process of developing new chemical antibiotics; (xiv) they can be a safe therapeutic option for patients allergic to antibiotics; (xv) antimicrobial treatment may be effective with the use of only a small phage concentration; and (xvi) phage particles readily cross the blood brain barrier and can be used to treat bacterial infections of the central nervous system (Sulakvelidze et al., 2001; Weber-Dabrowska et al., 2003; Matsuzaki et al., 2005; Skurnik and Strauch, 2006; Hermoso et al., 2007; Abhilash et al., 2009; O'Flaherty et al., 2009; Hagens and Loessner, 2010; Kutter et al., 2010; Wittebole et al., 2013).

However, phage therapy may also present several disadvantages, namely: (i) it is necessary to identify, in a first stage, the infectious agent responsible for the infection and then isolate its specific phage from the environment; (ii) when administered systemically, phage particles (which are protein entities) trigger an immune response leading to the production of antibodies, which can drastically reduce the effectiveness of the antimicrobial treatment; (iii) actually, only lytic phages may be used in phage therapy, which reduces the number of available phages eligible to integrate this therapy; (iv) it has to be defined which is the best route of administration, the optimal dose, the frequency of administration and the average duration of treatment with these biological entities; (v) new genes in some bacteriophages are being identified in a regular basis, and researchers are still unaware of what is their function; (vi) plain bacteriophage particles are prone to recognition by the immune system and thus are easily eliminated from the body, resulting in a drastic reduction of therapeutic efficacy; and (vii) bacteria developed many different types of mechanisms that confer resistance to phages (Hermoso et al., 2007; O'Flaherty et al., 2009; Parracho et al., 2012; Chan and Abedon, 2012; Wittebole et al., 2013). Phages are protein-based entities totally devoid of metabolic machinery that can potentially interact with the immune system within the human body, can actively replicate only in the presence of viable host bacterial cells, and can even evolve during manufacture or use, but are far from being unique in these regards (Loc-Carrillo and Abedon, 2011). For example, many protein-based (bio)pharmaceuticals can stimulate the immune system, chemical antibiotics that lyse bacteria will release *in situ* bacterial (endo)toxins, and live-attenuated vaccines both actively replicate and evolve within the body tissues. Protein-based drugs, chemical antibiotics, and whole vaccines have previously been approved for use despite these various properties. There are no antimicrobials displaying selective toxicity that will affect all possible microbial targets. Typically the narrowness of phage host ranges – few strains or species of bacteria – will at a minimum place limitations on presumptive treatment, *i.e.*, treatment courses that begin prior to the identification of the pathogen's susceptibility to antibacterials such as to specific phages. However, as phages can often be employed in combination with other antibacterial agents, including other phages (so-called phage cocktails), the lytic spectrum of phage products can be much broader than the spectrum of activity of individual phage types (Loc-Carrillo and Abedon, 2011). Even phage cocktails with broad spectrum of action are normally more selective in their spectrum of activity than typical "narrow-spectrum" antibiotics, a property that can be viewed as an additional advantage of phage therapy. When using bacteriophage particles as antimicrobial agents, the general aim should be to identify those bacteriophages that display good primary pharmacodynamics (*i.e.*, antibacterial virulence), minimal secondary pharmacodynamics (*i.e.*, low potential to do harm to patients), and good pharmacokinetics (*i.e.*, an ability to reach target bacteria *in situ*). Bacteriophage particles that do not adequately comply with these criteria should in most circumstances not be used as antimicrobial therapeutics (Loc-Carrillo and Abedon, 2011). Additionally, since phage particles are intrinsically proteins in nature and may eventually interact with the immune system of the body, a solution to surpass this apparent disadvantage may lie in protecting such phage particles via encapsulating such biomolecules within nanocarriers, eventually endowed with characteristics of invisibility towards the digestive and immune systems, or binding them to macroscopic supports thus rendering them insoluble. Combined, these strategies promote their structural and functional stabilization (Balcão and Vila, 2015).

2.4. Bacterial resistance to bacteriophages and bacteriophage kinetics

The mechanisms of bacterial resistance to bacteriophages are generally associated to the disruption of the phage adsorption process, through bacterial mutations that commonly lead to the loss of specific cell surface receivers that allow linking between bacterium and phage. Bacteria can also induce the production of layers rich in mucilage lining the entire bacterial surface, preventing contact of the phage with its respective receptor (Wittebole et al., 2013).

Additionally, bacteria may acquire resistance via lysogenic phages containing in their genetic material sequences that encode bacterial resistance or toxins and, after integration of the phage's genetic material into the genome of the bacterium, begin to acquire such resistance. In addition to these mechanisms, bacteria may hydrolyse the genetic material of the phage by restriction endonucleases present in their cytoplasm, and are also able to methylate their own DNA, working as a defense mechanism against phages.

The bacterial resistance can also be caused by mutations in genes that encode proteins either essential for phage replication or necessary in the assembly of new virion particles (Skurnik and Strauch, 2006; Wittebole et al., 2013).

However, bacterial resistances are not always supportive for the bacterium, because what usually happens is that the resistance may reduce the performance of the bacterium or, if the specific phage receptor is a virulence factor, the mutation may cause a drastic reduction in its virulence (Levin and Bull, 2004; Skurnik and Strauch, 2006).

Concerning phage "pharmacokinetics", this allows to establish the amount of phages that are available at the infection target site, in order to perform a therapeutic action, while describing the impact of the body on phages. This is actually a rather complex parameter, both due to the self-replicating nature of phages and because only a few studies have been produced. It is important to note that the process of *in vitro* replication of bacteriophages can be quite different from what actually happens *in vivo*, as the *in vivo* "pharmacokinetic" and "pharmacodynamic" processes differ from phage to phage. Thus, pharmacokinetics in phage therapy is very different from that associated with conventional antibiotic chemotherapy, and presents a number of critical parameters that must be considered: (i) the adsorption rate; (ii) the latency period; (iii) the initial phage dosage; (iv) the critical time; (v) the clearance rate; (vi) the ability to replicate phage particles *in situ*; (vii) the anatomophysiology of the host; (viii) the environmental conditions; and (ix) the phage distribution, which will largely depend on the individual's immune system (Skurnik and Strauch, 2006).

2.5. Applications of phage therapy

Over the last three decades, several studies have been developed involving the use of bacteriophages, which have clearly demonstrated the ability to infect pathogenic bacteria both in animals and humans, for example, the recently discovered φNK5 lytic phage, which is highly effective against *Klebsiella pneumoniae* in mice (Hung et al., 2011). Nowadays, there are some experimental models which aim at the administration of bacteriophages by either intravenous or intraperitoneal paths, and which exhibit therapeutic efficacy. However, the intravenous path is not ideal because it is not possible to ensure that bacteriophage solutions are completely pyrogen-free (Maura and Debarbieux, 2011). As the intravenous administration of phage solutions is no more the first option, a group of researchers attempted to orally administer a phage cocktail (containing phages SP15, SP21 and SP22) for the treatment of gastrointestinal infections caused by *Escherichia coli* O157:H7 in mice, and the number of bacteria detected in the faeces was substantially reduced. However, and despite the fact that strictly lytic bacteriophages are highly specific for their target bacterial hosts, the consequences to the intestinal flora and the resulting immune response are still unknown (Tanji et al., 2005).

Another route of administration has been studied and developed by several researchers, based on a topical model, in which bacterial infections in the skin can be treated with a simple external (topical) application of a cream containing bacteriophage particles. However, as in the case of oral administration, the adverse effects are still largely unknown, therefore making it necessary to deepen the knowledge regarding such model (O'Flaherty et al., 2005). More recently, the intranasal route of administration was selected for the treatment of pulmonary infections caused by *Pseudomonas aeruginosa* or by *Klebsiella pneumoniae* through the PAK-P1 phage and the SS phage, respectively. However, this approach has revealed a relatively low therapeutic efficacy compared to intraperitoneal administration, which was confirmed by a comparative study performed by Carmody et al. (2009).

In this context, another study proved that a single intranasal instillation containing PAK-P1 bacteriophage was sufficiently effective to prevent infection for a period of 24 h. The same result was obtained, but using phage P3-CHA, which is able to eradicate and prevent acute pulmonary infections caused by multidrug-resistant *Pseudomonas aeruginosa* isolated from a cystic fibrosis patient in a hospital in France (Chhibber et al., 2008; Debarbieux et al., 2010; Maura and Debarbieux, 2011; Morello et al., 2011).

In 2009, the FDA authorized a phase I clinical trial involving the use of a phage cocktail applied in venous leg ulcers, aiming at the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli*. The results of this clinical trial have shown that the aforementioned phage cocktail was safe and highly effective, when compared with the control group (Rhoads et al., 2009; Maura and Debarbieux, 2011). Also in 2009, a phase II clinical trial was conducted in England by the startup company Biocontrol Ltd., which demonstrated the efficacy of phage therapy in the treatment of chronic otitis caused by multidrug-resistant *Pseudomonas aeruginosa* (Wright et al., 2009; Maura and Debarbieux, 2011).

More recent studies indicate that a number of phage particles between 10^2–10^3 plaque forming units (PFU) is adequate to induce replication and therapeutic action at bacterial infections with 10^6–10^9 colony forming units (CFU) per millilitre (Rhoads et al., 2009; Wright et al., 2009; Parracho et al., 2012).

In the aforementioned clinical trials, liquid preparations containing bacteriophages were used and, for these to be effective and used with clinical purposes, it is necessary to promote standardization of the methodologies used, as well as to control the final quality of these products. Thus, there must be an adequate identification and monitoring of bacteriophage preparations for clinical applications, through the combined use of various analytical techniques such as: (i) DNA sequencing; (ii) scanning/transmission electron microscopy; and (iii) Polymerase Chain Reaction. All bacteriophages used in clinical trials conducted in humans must be strictly lytic and duly identified via the referred analytical techniques. The liquid bacteriophage preparations must exhibit adequate stability, and the storage conditions of the preparations should be optimized – the product must not be exposed to extreme variations in temperature, pH and high relative humidity. All phage preparations used in clinical trials should be duly sterilized through procedures properly validated and stipulated by the European Pharmacopoeia. All these parameters are essential to ensure the safety, efficacy and purity of phage preparations (Yang et al., 2010; Jończyk et al., 2011).

In the long term, it is expected to occur a renovation of the current system that is used by the pharmaceutical industry, since the model applied until now is not either the most suitable or compatible one with phage therapy. In this context, two models were set up that may be used in phage therapy: (I) the "conventional medicinal product development", or (II) the "traditional tailor-made approach". Therefore, at present, the scientific community is questioning whether it would be more profitable to produce phage preparations on a large scale and sell them as conventional drugs, involving high production costs and taking several months or even years to be developed, as happens with the aforementioned model (I), or to opt for a rational, personalized and flexible model performed in hospitals, as is the case with model (II), involving reduced costs and requiring only a few days or weeks (Merabishvili et al., 2009; Kutter et al., 2010; Pirnay et al., 2011, 2012).

Phage therapy is not exclusively directed towards health care, but is also targeted for the food industry. Despite being a "brain teaser" in the production of fermentation products such as cheeses and yoghurts, as phages destroy the bacteria necessary for the production of these dairy products, phages are used to destroy foodborne pathogens. There are several phage products available in the market, duly approved by the FDA, for the purpose of controlling bacterial infections/contaminations in food production processes.

With the availability of these products, at European level (*e.g.* Listex P100™), the number of infections by *Listeria monocytogenes* has stabilized (Hagens and Loessner, 2010).

Presently, there are phage preparations against several types of bacteria (such as *Escherichia coli* and *Salmonella* sp.) to be applied in animal foods and administered before cattle and chickens are killed in slaughter houses (Johnson et al., 2008). Currently, Intralytix Inc., a biotechnology company focused on the production and marketing of bacteriophage-based products targeted to control bacterial pathogens in environmental, food processing, and medical settings, has developed countless phage preparations used in the control of bacteria responsible for contaminating foodstuffs, namely: (i) ListShield™, for the control of listeriosis (approved by the FDA in August 2006); (ii) EcoShield™, used to prevent food contamination by *Escherichia coli*; and (iii) SalmoFresh™, which reduces contamination caused by *Salmonella* in food and was very recently considered a generally regarded as safe (GRAS) substance by the FDA (Atterbury, 2009; Balogh et al., 2010; Hagens and Loessner, 2010).

As examples, TechnoPhage (http://www.technophage.pt) and Innophage (http://www.innophage.com) are two Portuguese startup companies that promote research and development of new products based on the unique properties of bacteriophages, directed to the treatment, diagnosis and prevention of bacterial infections within the community, hospital and food industries.

Recent technological advances in this field open the door to the possibility of customizing bacteriophages and improve their characteristics, particularly: (i) expand the ability of bacteriophages to penetrate bacterial biofilms; (ii) enlarge their potency and effectiveness; (iii) adapt the spectrum of activities of bacteriophages to infections caused by numerous bacterial species and strains; and (iv) make them more stable and specific (Lu and Collins, 2009; Pouillot et al., 2010; Soto and Ratna, 2010; Maura and Debarbieux, 2011).

In the United States, there is now the possibility of phage particles being integrated in wastewater and potable water treatment systems. The process that is still being studied involves filtering the phages present in the wastewater through nylon filters, and subsequently apply them in wastewater and drinking water, contributing to the increase of their quality and the promotion of public health in general, preventing the spread of multiresistant bacteria (Tamaki et al., 2012; Potera, 2013).

3. Lysins and lysin therapy

Lysins are enzymes produced by bacteriophages that digest the bacterial cell wall and allow the release of the prophage, thus ensuring that new infection cycles are performed. Lysins have been widely tested and applied in various animal models, for the control and treatment of bacteria resistant to conventional chemical antibiotics (Fischetti, 2008; Schmelcher et al., 2012; Schuch et al., 2013).

Small amounts of purified recombinant lysins, exogenously administered in Gram-positive bacteria, showed a high therapeutic efficacy and promoted a quick lysis of the target bacterium. However, the antibacterial ability is limited, because if the lysins are externally applied only Gram-positive bacteria are affected, since these bacterial cells have no external membrane (Fenton et al., 2010). Lysins showed high reliability, specificity and quick ability to promote cell lysis in target bacteria, at mass concentrations in the order of nanograms, strongly reducing the number of microorganisms present, seconds after the addition of the lytic enzyme (Fischetti, 2008; McGowan et al., 2012).

Unlike chemical antibiotics, and as phages, phage lysins are selective and can be used to combat certain bacterial species or specific genus, not affecting the commensal flora, which is their main advantage (Hermoso et al., 2007; Fischetti, 2011; McGowan et al., 2012; Xu et al., 2012).

Lysins, which demonstrate therapeutic antimicrobial activity, are classified according to their catalytic activity (see Fig. 3), as: (i) *N*-acetylmuramoyl-L-alanine amidase (NAM-amidase); (ii) *N*-acetylmuramidase (muramidases or lysozymes); (iii) *endo*-β-*N*-acetylglucosaminidase (glucosaminidase); (iv) endopeptidase (including L-alanoyl-D-glutamate endopeptidase); and (v) lytic transglycosylase. In all these types of lysins, the most common ones synthesized by phages are amidases and muramidases (Hermoso et al., 2007; Fischetti, 2010).

After phage replication occurs inside the bacterial cell host, the cell wall needs to be broken down (lysed) in order to release the newly formed virions and ensure the performance of new infectious cycles. Over the years, phages developed two different strategies that allow the release of the phage progeny from the bacterium. The phages with double-stranded DNA developed lytic enzymes (lysins or endolysins) that accumulate in the cytoplasm of the bacterium during the last stage of the lytic cycle and have the ability to hydrolyse the peptidoglycan and cause cell lysis. Lysins are highly effective and evolved over thousands of years, with the main purpose of promoting bacterial lysis. As these hydrolytic enzymes do not have signal sequences (except in the case of lysin gp61, which has a signal sequence that allows itself to go directly to the periplasmic space and act on the cell wall), they cannot cross the cytoplasmic membrane and attack its substrate present in the peptidoglycan layer, and hence they require the help of other phage products, such as holins. Holins are hydrophobic proteins that promote bacterial membrane disruption, by inducing the formation of pores in the inner membrane and thus allow lysin to be exported through the cytoplasmic membrane to the cell wall, so that they may act (Wang et al., 2003; Borysowski et al., 2006; Hermoso et al., 2007; Stojković and Rothman-Denes, 2007; Fenton et al., 2010; Fischetti, 2010; McGowan et al., 2012; Schuch et al., 2013).

3.1. Lysin structure

Lysins from phages with DNA and that infect Gram-positive bacteria usually have a molecular weight ranging from 25 kDa to 40 kDa, except for PlyC, that is specific for *Stretococcus* sp. and have an approximate molecular weight of 114 kDa. The peptidoglycan layer is composed of several chains of sugars, *N*-acetylglucosamine and *N*-acetylmuramic acid, linked by β-1,4-glycosidic bonds, and tetrapeptide chains bonded to the lactyl group of the muramic acid via amide bonds (Donovan et al., 2006).

Lysins are produced and accumulated in the bacterial cytoplasm at the terminal phase of the phage lytic cycle. Generally speaking, since there are some exceptions as is the case with PlyC, lysins are enzymes composed of two protein domains: the catalytic domain, present in the N-terminal region, characterized by providing catalytic activity to the enzyme; and the binding domain, present in the C-terminal region and that allows a connection between the enzyme and the bacterial cell wall. Both domains are separated by a linker (see Fig. 4). The catalytic domain (present in the N-terminal region) is less variable compared to the binding domain (present in the C-terminal region), which may impart different catalytic activities depending on the type of bonds to be hydrolysed (Hermoso et al., 2007; Fischetti, 2008, 2010; Schuch et al., 2013).

The N-terminal region may be formed by: (i) *N*-acetylmuramidases (muramidases or lysozymes); (ii) *endo*-β-*N*-acetylglucosaminidases (glucosaminidases); (iii) lytic transglycosylases that cleave glycosidic bonds present in the peptidoglycan layer; (iv) endopeptidases that hydrolyze the peptide bonds of the peptidoglycan layer; or (v) *N*-acetylmuramoyl-

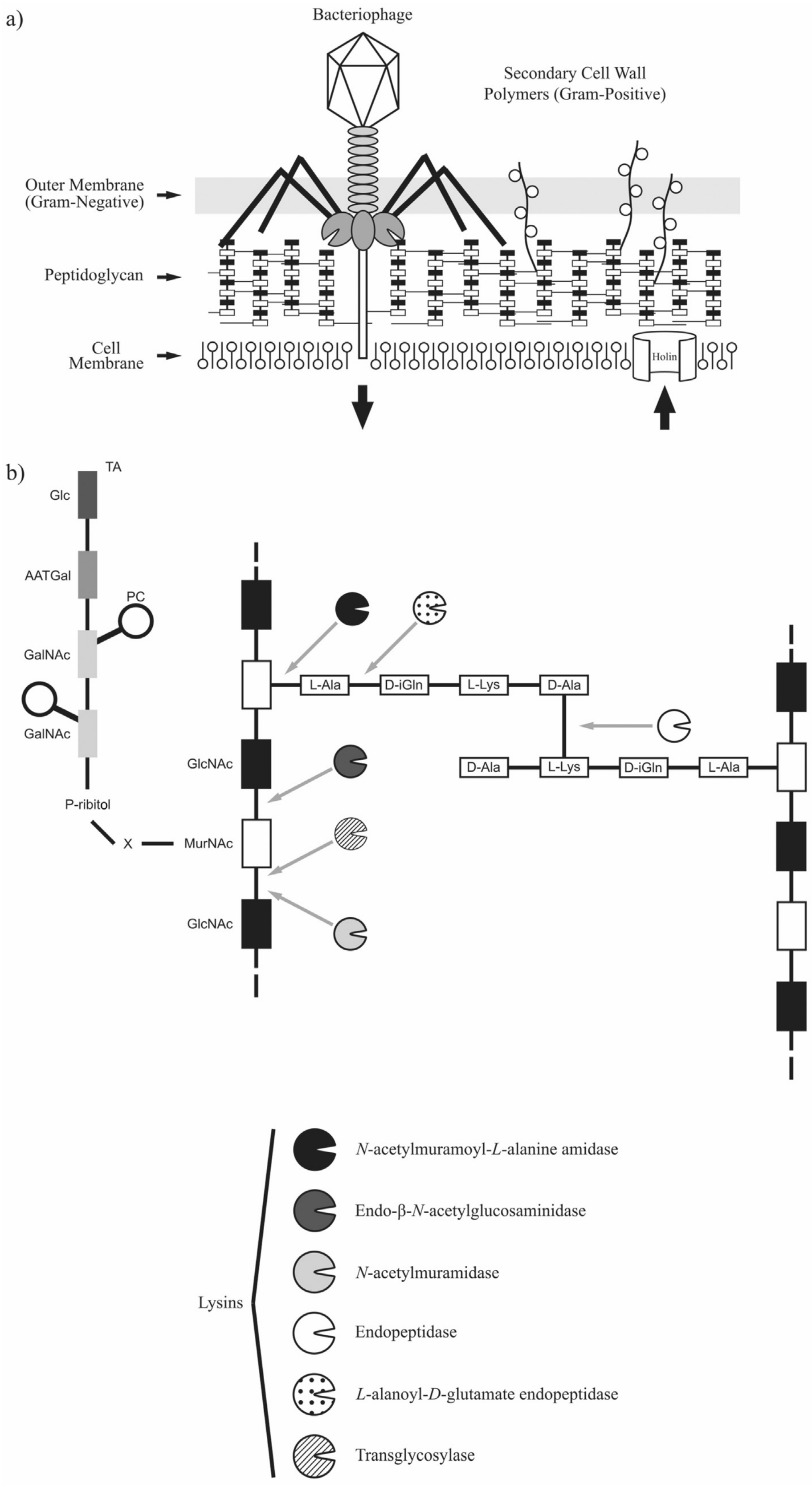

Fig. 3. Schematic representation of the bacterial cell wall (a) and of the several types of lysins, their mechanism of action and target in the host cell peptidoglycan (b). Source: adapted from Hermoso et al. (2007).

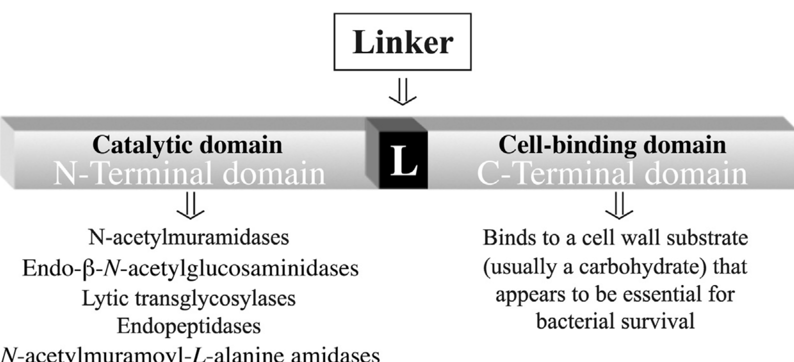

Fig. 4. Basic structure of lysins, characterized by having two protein domains, N-terminal and C-terminal domains, separated by a "linker".
Source: adapted from Fischetti (2008).

L-alanine amidases (amidases) that hydrolyze amide bonds (Hermoso et al., 2007; Fischetti, 2008, 2010).

Typically, each lysin has only one type of catalytic activity, however, bifunctional lysins were discovered, which possess two independent catalytic domains, capable of catalyzing the cleavage of more than one peptidoglycan bond, as are the cases with (i) phage B30 lysin, composed by muramidase and endopeptidase; (ii) phage φ11 lysin, specific for *Staphylococcus aureus*, containing endopeptidase and *N*-acetylmuramoyl-L-alanine amidase, indispensable for its activity to eliminate *Staphylococcus aureus* NCTC8325 biofilm; and (iii) phage K1-5 lysin, that has at its tail a K5 lyase protein and a *N*-acetylneuraminidase (endosialidase), which confers to this phage lysin the ability to act on K1 and K5 strains of *Escherichia coli* (Scholl et al., 2001; Hermoso et al., 2007; Sass and Bierbaum, 2007).

The C-terminal region of the lysins is characterized as being the binding domain to the cell wall (CBD), promoting the binding of lysins to the bacterial host cell wall, more precisely to the carbohydrates present there. This domain has a variable feature, due to the selection made over the years of evolution, with the purpose of recognizing specific bacterial hosts. In order to promote the cell wall digestion and subsequent release of the newly formed phage progeny, it is necessary that the bacterium cell is sensitive to lysin and possess a specific substrate in its cell wall to the C-terminal region of the lysin. Nevertheless, although this domain is variable in relation to the catalytic domain, it is highly specific for a particular substrate present in certain bacteria and binds to components that are essential for bacterial viability, which substantially reduces the number of bacterial resistance and, at the same time, ensures the release of newly formed phage progeny into the extracellular environment (Fischetti, 2008, 2010; Schuch et al., 2013).

In addition, researchers began to realize that different enzymatic domains could be exchanged in lysins, through genetic engineering, giving them simultaneously different specificities for other bacteria and different catalytic activities, leading to the development of chimeric lysins. Hence, some studies show that the catalytic domain of a lytic lysin specific for *Streptococcus pneumoniae* may swap with other domains and thus a new lytic enzyme is created with the same binding domain to the bacterial cell wall, also specific for the pneumococci domain but, at the same time, capable to cleave other types of bonds present in the peptidoglycan layer (Fischetti, 2008, 2010; Manoharadas et al., 2009).

Other scientific studies revealed that there is a synergism between oxacillin, a β-lactam antibiotic, and lysin ClyS, a chimeric enzyme synthesized from the fusion of the N-terminal domain of phage Twort lysin active against *Staphylococcus aureus*, and the C-terminal domain of the phage phiNM3 lysin, leading to protection of mice from infections caused by Methicillin-resistant *Staphylococcus aureus* (MRSA) (Fischetti, 2008; Daniel et al., 2010; McGowan et al., 2012).

According to McGowan et al. (2012), the most powerful and complex lysin identified to date is the phage PlyC lysin. This lytic enzyme is produced by phage C1 and consists of two protein domains, PlyCA and PlyCB, which have therapeutic activity exclusively against *Streptococcus* sp. from groups A, C and E, including *Streptococcus uberis*, and *Streptococcus equi*. Both protein domains are transcribed from two genes located on the same operon. The PlyCB is composed of 8 subunits which allows the bonding to the bacterium cell wall. PlyCA is responsible for the catalytic activity, and has two distinct catalytic domains, namely amidase and glycosidase. Thus, this endolysin can hydrolyze different types of peptidoglycan bonds present in the bacterial cell wall, making it the most potent lysin described so far. However, a small mutation in the catalytic domain can reduce the activity of this lysin by ca. 90% to 99% (Nelson et al., 2001, 2006; McGowan et al., 2012).

Bacteria have also developed a range of lysins named as autolysins, which hydrolyze the β-(1,4) bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of its own bacterial cell wall, and which are involved in the cell division processes. When produced in high concentrations, autolysins may induce cell death due to the difference of osmotic pressure between the bacterium and the external environment (Hermoso et al., 2007).

3.2. Advantages, disadvantages and limitations of antimicrobial therapy with lysins

There are several advantages associated to the use of lysin therapy, compared to conventional antimicrobial chemotherapy, namely: (i) phage lysins are selective and have a limited spectrum of action, so they may be used to fight only certain bacterial species or genus, not affecting the commensal flora; (ii) dilute concentrations of lysin preparations will be enough to reduce exponentially the number of viable bacteria, seconds after application of the lytic enzyme; (iii) to date, no bacterial resistance to these enzymes has been reported, due to the co-evolution process of billions of years established between phages and bacteria, leading to the development of a C-terminal region highly specific to molecules present in the wall of the host and that are essential to its viability; (iv) several studies have shown that there is a synergism between antibiotics and lysins; (v) bacteriophages are the most abundant entities on the planet, therefore there is a considerable number of lysins available for therapeutic applications; (vi) a number of pre-clinical tests *in vivo* have shown that antimicrobial therapy with lysins do not produce disturbing side effects; (vii) when applying different lysins possessing the same binding domain and different catalytic activities, there is a synergism between them, hence optimizing the therapeutic activity and, at the same time, reducing the possibility of resistant bacterial strains to emerge; (viii) there are already chimeric lysins active against MRSA strains; (ix) lysins exhibit a

high antibacterial activity, even against antibiotic-resistant bacteria; (x) unlike penicillin and cephalosporin, which inhibit the peptidoglycan synthesis and induce lysis of bacterial cells that are in the phase of cell division, lysins destroy the peptidoglycan directly, causing osmotic lysis of all specific bacteria; (xi) lysins are thermostable and can withstand temperatures up to ca. 60 °C; and (xii) lysins have simple synthesis processes and can be synthesized and purified in high quantities at a reduced cost, rendering them an excellent and innovative antimicrobial therapeutic strategy (Jado et al., 2003; Loeffler et al., 2003; Matsuzaki et al., 2005; Hermoso et al., 2007; Fischetti, 2008, 2010; Daniel et al., 2010; Fenton et al., 2010; Wu et al., 2012; Rodríguez-Rubio et al., 2013).

Concerning the disadvantages and limitations associated with the application of these purified lytic enzymes in the treatment of (pathogenic) bacterial infections, these are correlated to the production of antibodies by the (human) host. Unlike chemical antibiotics, which are small and non-immunogenic, lysins are peptides that stimulate the immune response, regardless of the way in which they are administered in the body, leading to the formation of antibodies that can reduce the activity of the lysin *in vivo*. Nevertheless, several studies conducted in hyper-immunized mice against phage lysin Cpl-1 proved that antibodies raised against the enzyme only slow down its lytic activity, without blocking it, without causing side effects. It is thought that this effect is due to a greater specificity of the lysin for the bacterial cell wall than the affinity of the antibody to the lysin, which prevents the antibody from inhibiting the catalytic domain of the lysin molecule (Loeffler et al., 2003; Hermoso et al., 2007; Fischetti, 2008, 2010). Another drawback or limitation of the lysin-based antimicrobial therapy is that, as mentioned before, when lysins are administered exogenously, they are only active against Gram-positive bacteria, since Gram-negative bacterial cells possess an outer and impermeable membrane to these enzymes. But there is a lysin produced by a phage active against *Bacillus amyloliquefaciens*, that displays a lipophilic amino acid sequence in the C-terminal region, which allows it to cross the outer membrane and overcome this issue (Orito et al., 2004).

Another possible limitation of lysins is correlated with their narrow spectrum of action. Depending on the clinical situation, it may be necessary to broaden such spectrum by combining different types of lysins in order to prevent the development of bacterial resistance and infections caused by more than one bacterial species (Wu et al., 2012).

Several studies reported in the specialty literature have not found bacterial resistance to this innovative antimicrobial therapeutic strategy, even after 40 cycles of exposure to low concentrations of these lytic enzymes (Schuch et al., 2002; Fischetti, 2008, 2010). This can be explained, as stated above, by the co-evolution process of billions of years, established between bacteria and their natural predators (*i.e.*, phages), to ensure that the phage progeny would not be retained within bacterial cytoplasm, and for this purpose a C-terminal region was developed in lysin molecules, displaying a very high specificity towards essential molecules for bacterial viability. This rationale explains the fact that lysins specific to streptococci and pneumococci possess, as receptors on the surface of the bacterial cell, amino alcohol (choline) and rhamnose, respectively, which are essential to the viability of the bacteria and their growth. Finally, it is noted that this co-evolution mechanism converts the bacterial resistance into an extremely rare event, making therapy with lysins extremely advantageous over conventional chemical antibiotics (Hermoso et al., 2007; Fischetti, 2008, 2010; Rodríguez-Rubio et al., 2013).

In a quite recent study conducted by Rodríguez-Rubio et al. (2013), no bacterial resistance to lysins was detected even after 10 cycles of exposure to three types of lysins specific for *Staphylococcus aureus*, either in liquid culture or on agar plates, indicating that lysin therapy may be an excellent alternative antimicrobial therapy to combat infections caused by this bacterium.

3.3. Applications of lysin therapy

3.3.1. Lysins in medicine and biotechnology

Since lysins exhibit a high antibacterial activity because they hydrolyze covalent bonds present in the bacterial cell wall, promoting a rapid lysis of the bacterial cell, lysins may be a possible and feasible strategy in the near future for the treatment of various bacterial infections caused by (but not limited to) (i) *Enterococcus faecalis*, (ii) *Enterococcus faecium*, (iii) *Clostridium perfringens*, (iv) *Bacillus cereus*, (v) *Staphylococcus aureus*, and (vi) *Bacillus anthracis* (to prevent and control biological weapons such as anthrax caused by this pathogenic bacteria), via intraperitoneal administration of lysin PlyG produced by phage γ (Borysowski et al., 2006; Hermoso et al., 2007; Fischetti, 2008, 2010; Proença et al., 2012; Rodríguez-Rubio et al., 2013; Schuch et al., 2013).

Three studies performed in animals have shown that a single dose of lysins, administered topically, drastically reduces (even to levels below the limit of detection) the amount of bacteria present in oral, nasal and vaginal mucosa. Although lysins display a short half-life, between ca. 15–20 min, this timeframe is enough to guarantee the antibacterial action of these lytic enzymes. It is possible, however, to expand this timeframe of action via modification of the Fc region (crystallizable fragment, at the lower region of the antibody responsible for modelling the cellular immune response upon binding of antibody to antigen (*i.e.*, the lysin moiety)) of the antibodies that neutralize these enzymes (Nelson et al., 2001; Loeffler et al., 2003; Cheng et al., 2005; Hermoso et al., 2007; Fischetti, 2008, 2010).

Presently, several intensive studies have been conducted involving intraperitoneal administration of lysins, such as Dp-1 and Cp-1, directed against *Streptococcus pneumoniae*, not only because this bacterium exhibits a high degree of resistance to the chemical antibiotics available in the market, but also because it causes pneumonia, meningitis, septicemia and otitis in children and immunocompromised hosts (Hermoso et al., 2007; Fischetti, 2010).

In one of such studies, a group of mice was infected with *Streptococcus pneumoniae* and, one hour after infection, they were intraperitoneally administered with a *bolus* (administration of a drug in order to quickly raise its concentration in blood plasma) of 2.0 mg lysin Cpl-1. After 48 h of administration, it was found that only 20% of the animals survived and that these were free from the pathogenic bacterium, demonstrating that it would require multiple applications (diluted) of the same *bolus*, or a parenterally administered solution, in order to eliminate all pathogenic bacteria from the body of the animals artificially contaminated (Jado et al., 2003; Rashel et al., 2007; Fischetti, 2008).

Recently, lysins have been applied in the treatment of bacterial infections in poultry caused by *Clostridium perfringens*, responsible for causing food poisoning and necrotic enteritis. Initially, a group of researchers tried to apply a treatment containing phages but, as more than 50 pathogenic strains of these bacteria were discovered, it would be necessary to associate the phage preparations in a complex cocktail containing over 20–30 kinds of phages. These researchers opted, therefore, to administer a combination of a group of lysins in the form of an enzyme cocktail containing peptidases, amidases and lysozymes, producing in this way a more effective antimicrobial therapy (Volozhantsev et al., 2011; Potera, 2013).

3.3.2. Synergism between lysins and antibiotics

Presently, several lysins were isolated from active bacteriophages against *Streptococcus pneumoniae*, in particular Cpl-1 (a muramidase) and Pal (an amidase), which possess the same bind-

ing domain (C-terminal) to the peptidoglycan layer of the host, but exhibit different catalytic activities. These enzymes were administered intraperitoneally and concomitantly, in amounts of 2.5 μg, one hour after infection of mice with *Streptococcus pneumoniae*, and succeeded in hindering the spread of pathogenic bacteria. However, the same did not occur when these lytic enzymes were administered separately, even in amounts of 5.0 μg. This clearly indicates that these enzymes act synergistically when they are administered simultaneously *in vivo* and, thus, they increase the therapeutic efficacy compared to the use of only one type of lysin. Interestingly, other studies show that the use of two enzymes with different catalytic activities, with the aim of combating the same pathogen, can also slow down the infection and even significantly reduce the emergence of new bacterial strains resistant to lysin (Jado et al., 2003; Fischetti, 2008; Daniel et al., 2010). It is also important to note that the association of lysin Cpl-1, isolated from bacteriophages active against *Streptococcus pneumoniae*, with gentamicin and penicillin, demonstrated a synergism between the different therapeutic agents applied, causing a drastic reduction in the number of that bacterium (Jado et al., 2003; Fischetti, 2008; Daniel et al., 2010).

3.3.3. *Other applications of lysin therapy*

Lysins can also benefit the health of the animal, preventing the dissemination of zoonosis and even preventing the transmission of pathogenic bacteria in food. For example, Ply700 (a lysin with lytic activity against *Streptococcus* sp.) and LysH5 (a lysin with lytic activity against *Staphylococcus aureus*) both have the ability to inhibit the proliferation of bovine mastitis thus avoiding contamination of milk and cheese (Celia et al., 2007; Obeso et al., 2008; Fenton et al., 2010).

Another phage enzyme applied in this area is PlyC lysin, which can also act as a particular antiseptic against *Streptococcus equi* (a bacterium responsible for causing infections in horses) preventing the transmission and dissemination of this bacterium. Additionally, PlyC lysin proved to be advantageous relatively to chemical antiseptics since these may be toxic to the animals, harmful to the environment and quickly lose their activity (Hoopes et al., 2009; Fenton et al., 2010).

As already stated above, lysins can also be applied in the food industry, as is actually the case with lysin Ply3626, which exhibits lytic activity against *Clostridium perfrigens*, a pathogenic bacterium responsible for causing food poisoning and high costs in the world of poultry production (Zimmer et al., 2002; Fenton et al., 2010).

Finally, lysins can also be applied successfully as diagnostic tools in the study of certain bacteria, since the current methods used, for example, in the detection of infections caused by *Bacillus anthracis* in humans, are quite time demanding and slow to produce results, which hamper implementation of treatment. One result is the work developed by Schuch et al. (2002), who developed a method using lysin PlyG that, after its addition, degrades the bacterial cell wall releasing ATP from the bacillus, which is then detected by a sensor, providing a diagnosis within a timeframe of 15 min.

More recently, a study conducted by Schuch et al. (2013) revealed that, due to the fact that lysins can recognize specific molecules on the surface of bacterial cells, which are essential to their viability, lysins can be applied in the identification of specific pathogenic bacterial receptors and thus help to develop new molecules capable to inhibit the biosynthesis of these receptors and reduce exponentially the number of resistant bacterial strains and block their proliferation. Hence, these researchers used a PlyG lysin specific to *Bacillus anthracis* to identify a neutral polysaccharide composed of galactose, *N*-acetylglucosamine and *N*-acetylmannosamine. Subsequently, they discovered the 2-epimerase, an enzyme involved in the biosynthesis of that polysaccharide, and synthesized a specific inhibitor of the 2-epimerase, named Epimerox^{TM}. This new molecule showed promising results, both *in vitro* and *in vivo*, efficiently inhibiting the proliferation of *Bacillus anthracis* in mice.

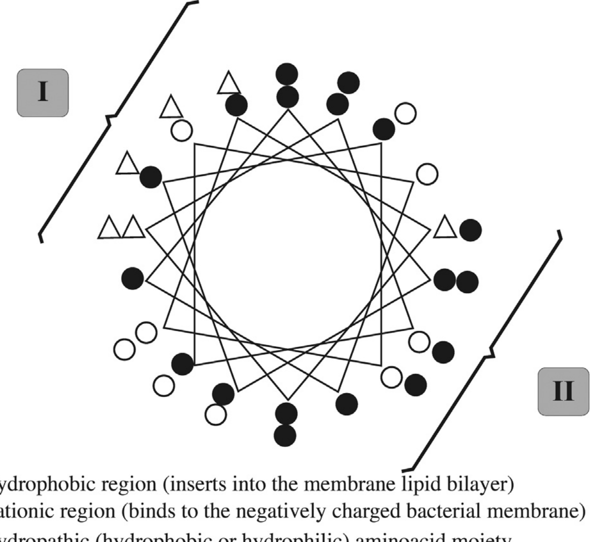

Fig. 5. Schematic basic structure of AMPs, showing a hydrophobic region that inserts into the bacterial cell membrane lipid bilayer and a cationic region essential for binding to the negatively charged bacterial cell membrane.
Source: adapted from Baltzer and Brown (2011).

4. Antimicrobial peptides

Antimicrobial peptides (AMPs) were first discovered back in 1922, when Alexander Fleming (1929) discovered lysozyme, an enzyme with antibacterial activity which was present in the tears and urine of humans. Currently, there are more than 1700 antimicrobial peptides known to man (Jenssen et al., 2006; Bruhn et al., 2011), some of which may be found in Table 2.

The galloping increase of bacterial resistance to chemical antibiotics and the possibility of these pharmaceutical compounds losing their effectiveness in the treatment of bacterial infections in the next five years, exponentially increased the interest of both researchers and pharmaceutical industries in the application of AMPs as therapeutic antimicrobial agents (Seo et al., 2012; Björn et al., 2012; Carlet et al., 2012b).

AMPs are polypeptides consisting of endogenous 12–50 aminoacids, synthesized via the ribosomal way. These peptides are ubiquitous in nature and generally possess a cationic (due to the presence of aminoacids such as cysteine and lysine, which are necessary for binding to the membrane) and amphipathic structure, having a hydrophobic and a hydrophilic domain, which is indispensable to promote the disruption of the bacterial cell membrane (see Fig. 5). AMPs exhibit a broad spectrum of action and constitute a defense strategy of both animals and plants against several types of bacteria, fungi and viruses (Zasloff, 2002; Baltzer and Brown, 2011; Fernebro, 2011; Laverty et al., 2011; Seo et al., 2012).

AMPs have various (perceived) functions, viz. (i) stimulate the accumulation of immune cells (neutrophils, macrophages and lymphocytes) at the site of infection, (ii) neutralize lipopolysaccharide endotoxins produced by Gram-negative bacteria, (iii) accelerate wound repair, (iv) stimulate angiogenesis, (v) control immune system responses against a particular microorganism, behaving as immunomodulators, and (vi) possess anti-inflammatory properties. Additionally, these endogenous polypeptides are active against multidrug-resistant bacteria. The extension of bacterial resistance

Table 2
Examples of several AMPs (antimicrobial peptides), considering their structure, number of disulfide bonds, source and mechanism of action.

Example of antimicrobial peptide	Three-dimensional structure	Peptide structure (ribbon model) obtained from the Protein Data Bank	Number of disulfide bonds	Source	Mecanism of action
Magainin	α-helix		---	Frog	Permeabilizes bacterial membrane
Cecropin A	α-helix	---	---	Silk moth	Membrane destabilizing
Mellitin	α-helix	---	---	Bee	Membrane destabilizing
LL-37	α-helix	---	---	Human	Membrane permeabilization
Buforin II	α-helix/extended	---	---	Toad	Binding of nucleic acid
α and β-Defensins	β-sheet		3	Mammals, with analogues in insects and fungi	Cell membrane and intracellular targets, inhibits macromolecular synthesis
Protegrin	β-sheet	---	---	Human, porcine	Very potent, membrane permeabilization
Polyphemusin	β-sheet	---	---	Horseshoe crab	Very potent, translocates into cells
Indolicidin	Extended		---	Bovine	Inhibits macromolecular synthesis and interacts with Ca^{2+} calmodulin
PR-39	Extended	---	---	Porcine	Inhibits DNA/RNA/protein synthesis, no pore formation
Thanatin	Loop structure		1	Insect (spined soldier bug, *Podisus maculiventris)*	Active against multiple Gram negative and Gram positive bacteria and fungi, but the mechanism underlying its bactericidal effect is still not understood
Defensin RTD-1	Cyclic		3	Monkey *(Rhesus macaques)*	Cell membrane and intracellular targets, inhibits macromolecular synthesis

Source: adapted from Jenssen et al. (2006) and Bruhn et al. (2011).

to these potentially therapeutic moieties tends to be reduced, due not only to the process of co-evolution between bacteria and these peptides over millions of years, but also to their mechanism of action, making them a possible alternative strategy to antibiotics in promoting the treatment of topical and possibly systemic bacterial infections (Bruhn et al., 2011; Laverty et al., 2011; Seo et al., 2012).

Although most of AMPs exhibit a cationic structure, some of them have an anionic structure rich in aspartic and glutamic acids, such as AMPs maximin-H5 (an anionic antimicrobial peptide from amphibians) and dermcidin (secreted by the human eccrine sweat glands and also acting as natural defense systems of the immune system of eukaryotic cells). These peptides can also adopt α-helical or β-sheet conformations, which provides them with an amphipathic structure, essential for the interaction with the bacterium cell membrane. However, anionic AMPs such as daptomycin, unlike cationic ones, require cations like zinc, which act as cofactors, essential for binding anionic peptides to the bacterium cell membrane (which is also anionic) and subsequent penetration, with consequent inhibition of ribonuclease, inducing cell death (Laverty et al., 2011).

4.1. AMP structures, properties and mechanisms of action

In structural terms, AMPs are (poly)peptides consisting of less than 100 amino acids encoded by single genes. They are synthesized during inflammation or in the presence of molecules produced by

pathogenic agents (Bruhn et al., 2011). AMPs are subdivided into four groups based on their secondary structure and amino acid composition: (i) peptides with a α-helical structure; (ii) peptides with a β-stranded structure – stabilized by two or three disulfide bridges, (iii) linear peptides that have a disordered structure in hydrophilic solutions but, under physiological conditions and hydrophobic environments, after contact with the lipid bilayer membrane they acquire an α-helix amphipathic structure, and (iv) peptides with a loop structure. However, there are peptides that do not fall within this classification, such as those with a cyclic structure (see Table 2) (Jenssen et al., 2006; Baltzer and Brown, 2011; Bruhn et al., 2011; Cho et al., 2012; Nakatsuji and Gallo, 2012; Seo et al., 2012).

The type of structure of AMPs is directly correlated to their size, mechanism of action, hydrophobicity, net charge, and polar angle (Cho et al., 2012). The structure of amphipathic AMPs is a key feature essential to their mechanism of action, since the hydrophobic region is responsible for the interaction with specific lipids present in bacterial cell membranes (such as lipoteichoic acids, present in Gram-positive bacteria, and the phosphate groups of lipopolysaccharides (LPS) present in Gram-negative bacteria), whereas the hydrophilic region of the peptide is responsible for the interaction with the phospholipid heads or the lumen of the bacterial cell membranes (Jenssen et al., 2006).

It is essential to note that AMPs do not affect the eukaryotic cells, because their cell membrane is composed of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, cholesterol and ergosterol, with a neutral net charge, which prevents the AMPs from affecting eukaryotic cells, since they are repelled. Unlike eukaryotic cells, prokaryotic cells (in particular, bacteria) have a cell membrane with a negative net charge due to the presence of phosphatidylglycerol, cardiolipin and phosphatidylserine, which makes AMPs specific for their phospholipid bilayer. This significantly reduces any potential toxic effects on the host cells and, at the same time, contributes to a significant reduction of potential bacterial resistance to this innovative antimicrobial therapeutic strategy (Jenssen et al., 2006; Baltzer and Brown, 2011; Laverty et al., 2011).

Concerning the mechanism of action of AMPs, they promote, in general, the disruption of the cell membrane, via pore formation and subsequent cell lysis (see Fig. 6). Initially, there is an electrostatic interaction between the positively charged peptide and the negatively charged bacterial cell membrane. Subsequently, the peptide structure is inserted into the phospholipid bilayer membrane and then the peptide aggregates, leading to the formation of a complex structure that promotes the formation of pores in the membrane and consequent cell lysis (Cho et al., 2012). There are 4 putative mechanistic models that explain in detail the mechanism of action of different types of AMPs (see Fig. 6).

In the first model, known as the "aggregate" model, AMPs bind to the phospholipid bilayer via electrostatic interactions and, thereafter, becomes inserted into the membrane by promoting the formation of complexes between peptides and lipids thereby leading to the formation of pores without a specific orientation, with different sizes and shapes, which induce the depletion of ions (see Fig. 6A). In the second model, known as the "toroidal pore" model, and following the initial electrostatic interaction, the AMP reorients itself perpendicularly to the plane of the cell membrane. Then, the hydrophilic region of the peptide interacts with the phospholipid structure of the membrane and the peptidic hydrophobic region interacts with the lipid core, leading to the formation of a pore with a specific orientation (see Fig. 6B). In this model, the membrane suffers an invagination with a positive curvature, leading to the formation of a small hole, maintained by electrostatic repulsions of the heads of the phospholipids, in which the peptide is aligned (see Fig. 6B). In the third model, known as "barrel-stave" model, formation of pores in the membrane by the AMPs occurs through a barrel-shaped cluster; in a first phase, peptide binds to the membrane via electrostatic interactions and becomes inserted perpendicularly within the membrane, after which, in a second phase, while the hydrophobic region of the peptide structure binds to the lumen of the bacterial cell membrane forming the outer portion of the pore, the hydrophilic portion of the peptide makes the internal portion of the pore. The pore size depends both on the number of peptides in the aggregate and on the lipid characteristics of the membrane (see Fig. 6C). Finally, in the fourth model, known as the "carpet" model, some AMPs are aligned together in a position parallel to the membrane phospholipid bilayer, covering the location like a carpet. When the number of peptides in place reaches a certain concentration, an emulsion is formed which leads to the destabilization of the membrane, promoting the formation of micelles and pores in the bacterial cell membrane (see Fig. 6D) (Jenssen et al., 2006; Baltzer and Brown, 2011; Cho et al., 2012; Laverty et al., 2011; Seo et al., 2012).

It is important to note that not all AMPs act only by disrupting the cell membrane; some of them promote the death of bacterium cells without reaching the minimum effective concentration, since they are accumulated intracellularly. Such AMPs can inhibit essential processes for cell viability, viz. (i) DNA replication or mRNA synthesis, when AMPs reach their minimum inhibitory concentration, as it happens with buforin II, pleurocidin and dermaseptin; (ii) protein synthesis, by indolicidin and PR-39; (iii) rearrangement of protein, promoted when pyrrhocoricin binds to chaperone hsp70 and prevents proper protein folding, which leads to an accumulation of inactive proteins and subsequent cell death (pyrrhocoricin is a highly active antibacterial peptide isolated from insects, which inhibits chaperone-assisted protein folding via binding to the 70 kDa heat shock protein DnaK via its amino terminal half, whereas its C-terminus functions as an intracellular delivery module); (iv) activity of some anionic cytoplasmic enzymes, by AMPs with an extremely positive charge; and (v) lipid II transglycosylation, an essential process for peptidoglycan synthesis by lantibiotics (nisin and mersacidin) (see Fig. 6E–H) (Bencivengo et al., 2001; Jenssen et al., 2006; Baltzer and Brown, 2011; Cho et al., 2012; Laverty et al., 2011; Seo et al., 2012).

Recently, it was discovered that papiliocin induces the production of oxygen free radicals, which are responsible for damaging the DNA, the cell membrane and mitochondria, inducing apoptosis and consequent cell death. However, in the context of a bacterial infection, it is likely that AMPs exhibit more than one mechanism of action simultaneously (Cho et al., 2012).

4.2. Advantages and disadvantages of AMPs for antibiotherapy

In terms of the advantages of using AMPs as therapeutic agents in antibiotherapy, the following considerations must be taken into account: (i) the mechanism of action of AMPs is performed in essential components that are present in the bacterial cell membrane, compromising cell viability; (ii) several AMPs exhibit simultaneous antibacterial, antifungal and antiviral activity and, in cases of infections by multiple biological entities, only one therapeutic agent is effective; (iii) AMPs demonstrate therapeutic antimicrobial activity in extremely low amounts, in the order of micrograms and nanograms; (iv) AMPs exhibit a wider spectrum of action; (v) they are found in high concentrations in the site of action, *i.e.*, the site of infection; (vi) AMPs also exhibit anti-inflammatory activity; (vii) the probability that bacteria develop resistance to this new therapeutic approach is reduced; (viii) AMPs act very quickly; (ix) AMPs inhibit biofilm formation; (x) AMPs interact with bacterial cells which are, or not, in the process of cell division; and (xi) AMPs demonstrate synergism when administered in conjunction with chemical antibiotics (Gordon et al., 2005; Bruhn et al., 2011; Björn et al., 2012; Devocelle, 2012; Seo et al., 2012).

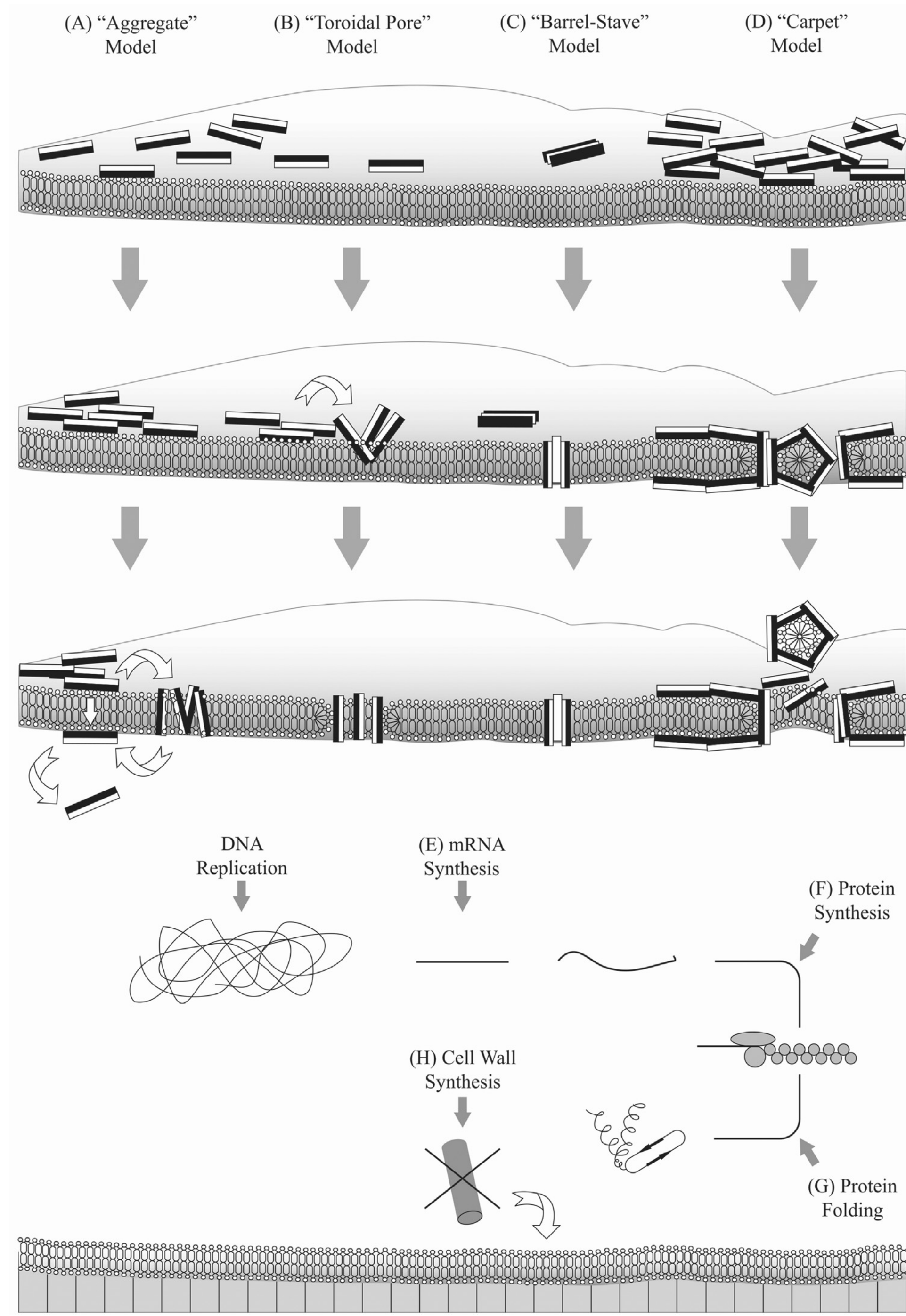

Fig. 6. The various putative mechanistic models for the action of AMPs.
Source: adapted from Jenssen et al. (2006).

However, when applied as therapeutic agents AMPs also demonstrate some disadvantages, viz. (i) they are susceptible to the action of proteases, easily losing activity, which may constitute a limiting factor when promoting the administration of AMPs to a systemic extent; (ii) AMPs can be cytotoxic for various host cells, namely when administered in high concentrations, and may destabilize cell membranes eventually leading to their disruption; (iii) AMPs are influenced by pH variations and may lose activity when administered in environments with low concentrations of salts or when interacting with plasma proteins; (iv) AMPs have high costs of production and purification; (v) AMPs can produce toxic effects both at a systemic and a topical extent; (vi) they may induce sensitivity and allergies after several applications; (vii) AMPs are not yet well studied from the "pharmacokinetics", "pharmacodynamics" and "toxicological" perspectives; and (viii) AMPs exhibit low stability due to the fact that they are mainly composed of L-aminoacids, thus being more susceptible to proteolysis (Gordon et al., 2005; Bruhn et al., 2011; Devocelle, 2012; Seo et al., 2012; Slaninová et al., 2012).

4.3. Bacterial resistances to AMPs

Back in 2002, Zasloff stated that bacterial resistances to this innovative antimicrobial therapeutic strategy would be

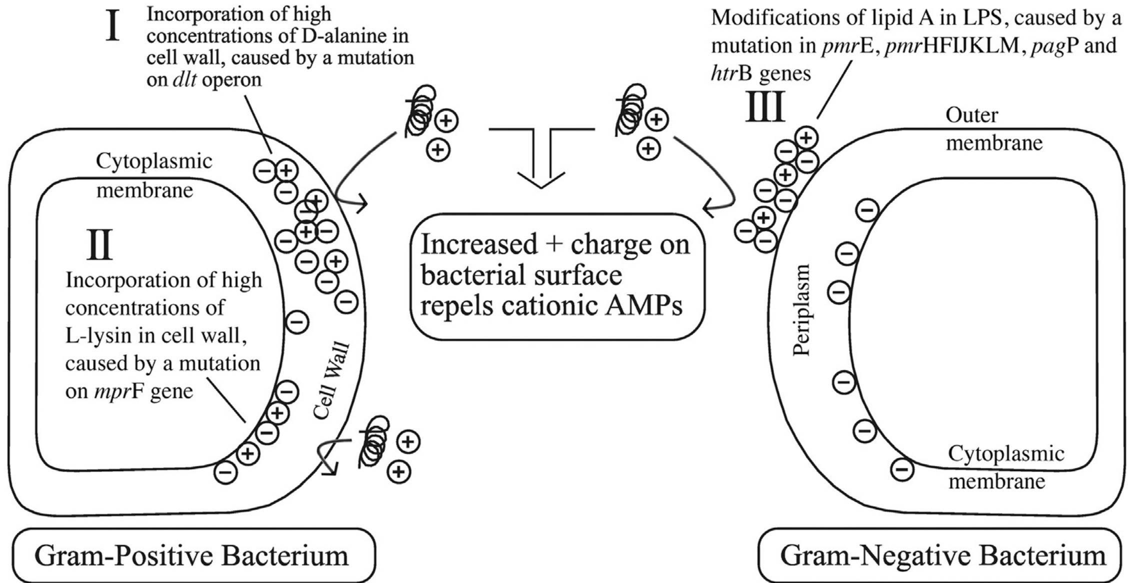

Fig. 7. Putative mechanism of both Gram-positive and Gram-negative bacterial resistance to AMPs mediated by changes in the net surface charge of the bacterial membrane. Source: adapted from Nizet (2006).

improbable, since AMPs have, as receptors, essential structures for bacterial viability.

However, a study conducted by Kristian et al. (2003) revealed that, a series of mutations in the operon *dlt*ABCD of *Staphylococcus aureus* that induced an overexpression of this operon, made this bacterium capable of neutralizing the negative charge of its own membrane via incorporation of high concentrations of D-alanine (D-alanylation process) and L-lysin (when mutation occurs in the *mpr*F gene). These mutations led to a reduction in the negative charge of the bacterial membrane, which is essential for the electrostatic interaction between the AMP and the bacterium, resulting in repulsion of the antibacterial peptide and subsequent resistance of the bacteria to the AMP, as can be seen in Fig. 7 (see Fig. 7I and II). It is important to mention that the same applies to Gram-negative bacteria, by modifying the LPS present in the outer membrane, comprising the incorporation of long chain fatty acid moieties and thus reducing membrane permeability to AMPs and increasing membrane stability.

The resistance to antimicrobial peptides is also directly correlated with (i) the introduction of 4-aminoarabinose (Ara4N) in lipid A phosphate groups (anionic dimmers of glucosamine linked to a fatty acid chain surrounded by phosphate groups) caused by mutations in genes *pmr*E and *pmr*HFIJKLM of Gram-negative bacteria (see Fig. 7III); (ii) the acetylation of lipid A caused by a mutation in genes *pag*P and *htr*B that causes a decrease in the permeability of the outer membrane of Gram-negative bacteria; (iii) a mutation in the gene *kas*B that promotes the synthesis of short chain mycolic acids in its outer membrane and drastically reduces its permeability to AMPs; (iv) a mutation in gene *emm*1 which stimulates the production of M1 protein that is found in bacterial cell wall and binds to AMP, blocking its mechanism of action (see Fig. 8I); (v) the production of extracellular proteases that synthesize dermatan sulfate, a glycosaminoglycan that stops the mechanism of action of α-defensins, as is the case with *Pseudomonas aeruginosa* and *Enterococcus faecalis* (see Fig. 8II); (vi) the synthesis of proteins that neutralize, or bind to, the AMP, preventing it from acting and, at the same time, preventing the minimum concentration to be reached so as to perform a bactericidal action, as is the case with staphylokinase from *Staphylococcus aureus*, which binds to α-defensins (AMP produced by human neutrophils) of the body and prevents them from acting (see Fig. 8III); (vii) the presence of efflux pumps that expel AMPs to the periphery of the membrane, preventing them from acting, as what happens with the efflux pump MtrCDE synthesized by *Neisseria gonorrhoeae* (see Fig. 9I); (viii) the synthesis of proteases that cleave the AMPs at the extracellular level, promoting their denaturation, as is the case with aureolysin, a metalloprotease synthesized by *Staphylococcus aureus* that inactivates LL-37 (see Fig. 9II); and (ix) formation of biofilms that reduce contact between bacteria and AMPs, hence preventing them from acting (Nizet, 2006; Baltzer and Brown, 2011; Laverty et al., 2011).

4.4. Potential applications of AMPs

There are numerous factors that make AMPs a potential and innovative alternative strategy to current conventional antibiotics, viz. (i) they may be employed as anti-infective agents; (ii) they may be applied simultaneously with several conventional antibiotics and antiviral drugs, and there may be even synergistic effects between them; (iii) they may be applied as immunomodulators, stimulating the immune system; and (iv) they neutralize bacterial endotoxins and prevent, for example, septic shock (Gordon et al., 2005; Baltzer and Brown, 2011; Kosciuczuk et al., 2012).

Despite the numerous advantages to establish themselves as a new and alternative therapeutic strategy to current antibiotics, few AMPs have been accepted by the FDA and the EMEA up to now. The application of AMPs, at the moment, is limited to topical formulations. To obtain the therapeutic antimicrobial effect in *in vivo* clinical trials, high concentrations of AMPs are required at the site of infection, but such high concentrations are very close to the toxic doses; additionally, AMPs have a small size and are filtered in the kidneys, which drastically reduces their half-life (Giuliani et al., 2007; Park et al., 2011; Yeung et al., 2011).

The first AMP developed was pexiganan, a synthetic analogue of magainin 2 (obtained from the African clawed frog, *Xenopus laevis*) consisting of 22 aminoacids, which was administered as a cream for topical applications, called Locilex™, in the treatment of feet ulcers in diabetic patients; however, it was not approved by FDA due to the lack of efficacy in phase II clinical trials (Giuliani et al., 2007; Park et al., 2011).

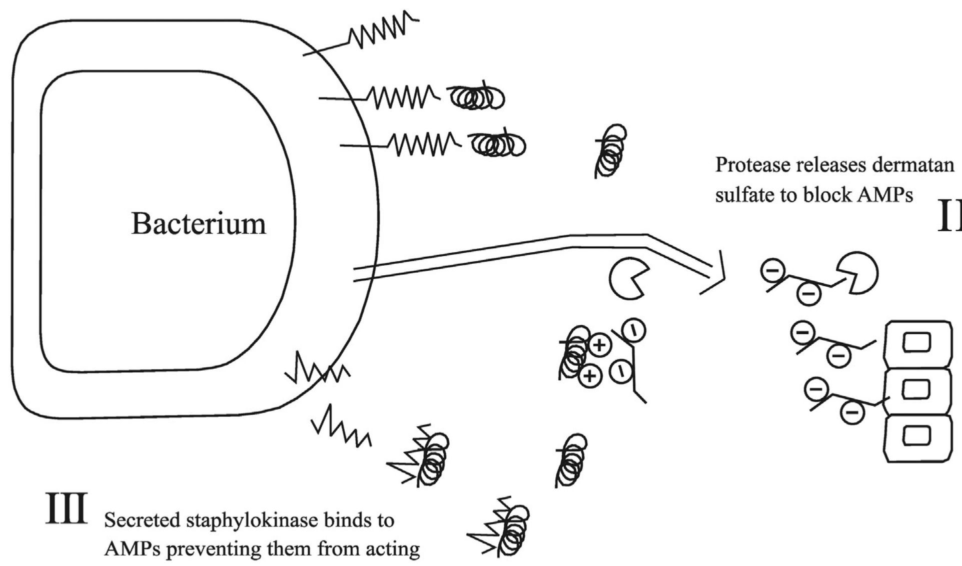

Fig. 8. Mechanism of bacterial resistance to AMPs via binding to surface proteins on the bacterial cell.
Source: adapted from Nizet (2006).

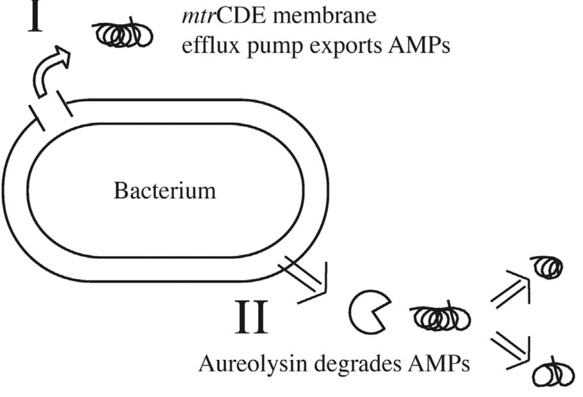

Fig. 9. Mechanism of bacterial resistance to AMPs via efflux pumps and aureolysin-mediated degradation.
Source: adapted from Nizet (2006).

Presently, there are several AMPs in both preclinical testing and clinical trials, which demonstrate various therapeutic activities, such as: (i) MBI-226 (omiganan) cationic peptide, a synthetic analogue of indolicidin with an extended spectrum of action, used in the treatment of blood infections caused by catheters; (ii) XOMA 629 and Migenix MX-226, active against *Propionibacterium acnes*, used in the treatment of acne; (iii) Migenix MX-226, which has anti-inflammatory activity since it suppresses the release of cytokines by the body, stimulated by the presence of *Propionibacterium acnes* in the skin; (iv) iseganan (IB-367), a synthetic cationic AMP with a broad spectrum of activity against bacteria and fungi, used in the treatment of respiratory infections, cystic fibrosis and mouth ulcers; (v) P113, a cationic peptide consisting of 12 aminoacids present in saliva, exhibiting a high therapeutic activity *in vitro* against *Candida albicans*, with potential to be used in the treatment of oral candidiasis in people infected with HIV; (vi) DPK-600 peptide, which is in phase IIa clinical trials for the treatment of atopic dermatitis; (vii) plectasin, the first AMP obtained from a fungus, *Pseudoplectania nigrella*, active against *Streptococcus pneumoniae* (McInturff et al., 2005; Mygind et al., 2005; Fernebro, 2011; Brandenburg et al., 2012).

At the moment, the AMPs accepted/approved by FDA and already on the market are polymyxins B and E (colistin) and daptomycin. Daptomycin is an anionic lipopeptide (a peptide associated with a lipid structure, which confers it therapeutic activity against bacteria and fungi) naturally produced by *Streptomyces roseosporus*. It is used to treat skin and mucosa infections caused exclusively by Gram-positive bacteria. It has been demonstrated that daptomycin may be applied in the treatment of infections caused by *Staphylococcus* sp. and endocarditis caused by *Enterecoccus* sp. and, in the United Kingdom, it has been already commercialized in the form of an injectable formulation called Cubicin® (Livermore, 2008; Yeung et al., 2011; Laverty et al., 2011; Brandenburg et al., 2012).

Polymyxins B and E are cationic lipopeptides discovered between 1940 and 1950, which were isolated from *Bacillus polymyxia* and have a cyclic structure associated with a lipid component in the N-terminal, responsible for their therapeutic antimicrobial activity. These cationic AMPs lie within phagocytes and at the surface of different epithelia in the body, acting as a natural defense mechanism. There are already available on the market several pharmaceutical formulations containing these lipopeptides, indicated in the treatment of skin and eye infections caused by Gram-negative bacteria. Polymyxin E, known as colistin, may also be administered parenterally in the form of colistin methanesulfonate, a prodrug indicated in the treatment of septicemia in cystic fibrosis, skin, urinary, and respiratory infections caused by *Pseudomonas aeruginosa* (Li et al., 2005; Cirioni et al., 2007; Yeung et al., 2011; Laverty et al., 2011; Brandenburg et al., 2012).

Relatively recently, Yan et al. (2011) discovered in the scorpion poison, the Hp1090, an AMP with a α-helical and amphiphilic structure, active against the hepatitis C virus (HCV). This virus is mainly responsible for causing chronic hepatitis, associated with cirrhosis and hepatocellular carcinoma and, until nowadays, there is no cure or vaccine against HCV. Thus, the Hp1090 appears as a potential therapeutic strategy because it inhibits replication of HCV RNA and prevents viral infection by directly damaging its membrane, at a 50% inhibitory concentration (IC_{50}) of 7.62 μg/mL (500 μM).

BmKn2, a peptide that has antibacterial activity, was recently isolated from the scorpion (*Mesobuthus martensii*) poison. Subsequently, BmKn2 originated a mutant peptide named Kn2-7, which exhibits greater therapeutic antimicrobial activity and reduced hemolytic effects, compared to the original BmKn2 peptide, since due to the substitution of aminoacids at its hydrophilic portion, it has a higher positive charge, which facilitates electrostatic interactions between the peptide and the bacterium cell wall (Cao et al., 2012). Application of the topical formulation containing AMP Kn2-7 in mice infected with *Staphylococcus aureus* promoted healing of the skin infection within a timeframe of 4 days, indicating that this is a potential therapeutic antimicrobial agent for external applications. Additionally, it was found that, besides being active against *Staphylococcus aureus*, AMP Kn2-7 is also active against several other Gram-positive bacteria such as *Bacillus subtilis*, *Bacillus thuringiensis* and *Micrococcus luteus*, Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*, and even against antibiotic multiresistant bacteria (Cao et al., 2012). According to these authors, Kn2-7 displays a greater activity than BmKn2, and the MIC for Kn2-7 to be used in the treatment of bacterial infections caused by Gram-negative bacteria is much higher than that for those caused by Gram-positive bacteria.

Additionally, according to Chen et al. (2012), peptide Kn2-7 also demonstrated to be the most active against HIV-1, with a half maximum effective concentration (EC_{50}) of 2.76 μg/mL, exhibiting a high tropism to HIV virus and effectively inhibiting its replication; however, and despite the fact that this study clearly shows that AMPs also possess anti-viral activity, the mechanism of action of Kn2-7 is not yet cleared.

Cationic AMPs may also be applied in the treatment of tumoral cells, since such cells possess in the external membrane high concentrations of phosphatidylserine and *O*-glycosylated mucins (3–7 times higher than those values for normal cells), giving them a negative surface charge and making them a potential target for cationic AMPs.

AMPs bind to the tumor cells via electrostatic interactions and induce pore formation in the cell membrane, promoting the release of cytoplasmatic electrolytes and subsequent cell death. Some cationic AMPs can induce apoptosis by stimulating the synthesis of oxygen reactive species, such as hydrogen peroxide, hydroxyl radicals and superoxide anions, which accumulate intracellularly. Subsequently, these peptide entities promote changes in the mitochondrial membrane, depolarization and release of cytochrome *c*, which induces the activation of caspase that, in turn, induces DNA and nucleus changes with concomitant changes in cell morphology and ultimately leading to cell death. There are a few examples, viz. (i) cecropin B, isolated from *Hyalophora cecropia*, which exhibits antitumor and antiproliferative activity in bladder tumor cells without affecting fibroblasts (control cells) following administration of 65 μM peptide; (ii) LL-37, which is present in the granules of neutrophils, involved in the mechanisms of innate immunity and immunomodulatory and anti-infectious activities; (iii) bovine lactoferrin, an iron carrier glycoprotein that inhibits proliferation of MDA-MB-435 breast tumor cells and THP-1 tumor cells involved in monocytic leukemia (Suttmann et al., 2008; Cho et al., 2012; Pushpanathan et al., 2013).

It is essential to note that AMPs can also be applied in the prevention of bacterial biofilm formation. The cationic peptide LL-37, apart from being applied in the treatment of tumoral cells, is also able to inhibit the formation of biofilms of *Pseudomonas aeruginosa* at a concentration of 0.5 μg/mL (Overhage et al., 2008; Amer et al., 2010).

4.4.1. Synergism between AMPs and antibiotics

AMPs may be associated with antibiotics, acting as a combination antibiotherapy. This synergism is due to the fact that this innovative therapeutic group causes a disruption in the bacterial cell membrane, thus increasing its permeability to antibiotics and facilitating their penetration and accumulation in the bacterial cytoplasm. As an example, one might refer the synergism established between magainin II and cecropin A when administered in association with rifampicin, substantially reducing the number of different strains of *Pseudomonas aeruginosa* not only in *in vitro* but also in *in vivo* clinical trials (Baltzer and Brown, 2011; Park et al., 2011).

Other studies revealed that the cationic peptide P5, administered concomitantly with isepamicin, promoted the disruption of bacterium cell membrane, which facilitated the penetration of the antibiotic into the intracellular environment and consequent inhibition of protein synthesis, making certain strains of *Pseudomonas aeruginosa* (which were once resistant to isepamicin) sensitive again to this aminoglycoside antibiotic (Cirioni et al., 2007; Jeong et al., 2010; Peters et al., 2010; Baltzer and Brown, 2011; Park et al., 2011).

To overcome the problems and disadvantages associated with AMPs, several methods have been proposed nowadays, including: (i) introduction of new aminoacid chains, amino or acetyl groups, in the terminal region of the AMPs in order to increase the peptide stability against the action of proteases; (ii) encapsulation of AMPs in liposomes, which are surface-decorated with antibodies, ensuring that they only accumulate exclusively in the desired site of action, hence reducing the side effects and toxicity at a systemic extent; (iii) synthesis, via genetic engineering, of AMPs with a high antimicrobial activity, at a reduced cost and simple synthesis processes, as is the case with AMP P-113, active against *Candida albicans, Candida glabrate, Candida parapsilosis and Candida tropocalis,* and hLF1-11 (human lactoferrin 1-11), active against MRSA, multidrug-resistant *Acinetobacter baumannii* and *Candida albicans* (Gordon et al., 2005; McPhee et al., 2005; Samad et al., 2007; Laverty et al., 2011; Seo et al., 2012; Yount and Yeaman, 2012).

5. Antibiotherapy with bacteriocins

In 1925, the first bacteriocin was discovered by André Gratia and became known as colicin V (Gratia, 1925; Gratia, 2000). This investigator noticed that the antimicrobial compound produced by a strain of *Escherichia coli* V ("verotoxin-producing strain", that is, virulent) prevented the growth of other strains of *Escherichia coli* (Gillor et al., 2005; Nishie et al., 2012).

Bacteriocins are recognized as a subgroup of AMPs, encoded in the ribosomal DNA or plasmids, and are synthesized by bacterial ribosomes, inhibiting the growth of bacteria of the same species or the same genus (Nissen-Meyer et al., 2009; Nishie et al., 2012; Kawada-Matsuo et al., 2013). Bacteriocins can be used by bacteria as a defense strategy against other highly specific and common bacteria, eliminating potential opponents and increasing the number of nutrients available in the environment for its own growth. There are many types of bacteriocins, with different structures and, consequently, different mechanisms of action (Lee and Kim, 2011).

This subgroup of AMPs can also be synthesized by Gram-negative bacteria. The most known and studied bacteriocins are colicins and microcins, which are synthetized by *Escherichia coli* and inhibit the growth of Gram-negative bacteria such as *Aeromonas* sp., *Escherichia* sp., *Salmonella* sp., *Yersinia* sp. and *Pseudomonas* sp. On the other hand, Gram-positive bacteria can synthetize, for example, subtilin and nisin A (Izadpanah and Gallo, 2005). These bacteriocins are not active against Gram-negative bacteria, due to the presence of the outer membrane in these bacterial cells. Table 3 displays several examples of bacteriocins produced by both Gram-negative and Gram-positive bacteria, and their applications (Duquesne et al., 2007; Papagianni and Anastasiadou, 2009; Lee and Kim, 2011).

Table 3
Examples of bacteriocins produced by Gram-positive and Gram-negative bacteria, and their potential uses.

Bacteriocin	Producing bacteria	Potential uses	Type of bacteriocin
Lanthiopeptin	*Streptoverticillium cinnamoneum*	Treatment of Herpes simplex virus	Lantibiotic
Epidermin	*Staphylococcus epidermidis*	Treatment of skin infections	Lantibiotic
Lacticin 3147	*Lactococcus lactis*	Treatment of mastitis infections	Lantibiotic
Mersacidin	*Bacillus subtilis*	Treatment of infections by vancomycin resistant strains	Lantibiotic
Mutacin	*Streptococcus mutans*	Treatment of dental cavities	Lantibiotic
Nisin	*Lactococcus lactis*	Treatment of peptic ulcers; inhibition of multidrug resistant pathogens; antimicrobial barrier in implanted medical devices	Lantibiotic
E1, E4, E7	*Escherichia coli*	Treatment of hemorrhagic colitis and hemolytic uremic syndrome	Colicin
24, J25, L	*Escherichia coli*	Treatment of salmonelosis	Microcin
B17	*Escherichia coli*	Antibacterial agent in cattle	Microcin
S-35	*Pseudomonas aeruginosa*	Treatment of pulmonary infections	Pyocin

Source: adapted from Gillor et al. (2005).

Nowadays, a freely accessible database is available, known as Bactibase, which contains information on more than 200 bacteriocins produced by many bacteria. This database can be used to optimize the application of bacteriocins not only in the food industry, as biopreservatives, increasing food safety, but also in the pharmaceutical industry, supporting researchers in the development of new bacteriocins or new pharmaceutical products with applications in medicine (Hammami et al., 2010).

Classification of bacteriocins is currently under review, however, these AMPs are broadly classified into four classes (see Table 4), according to: (i) chemical structure; (ii) molecular weight; (iii) mechanism of action; (iv) tropism to a specific receptor; and (v) percentage of modified aminoacids (Cotter et al., 2005; Oppegard et al., 2007; Nissen-Meyer et al., 2009; Nishant et al., 2011; Bodaszewska-Lubas et al., 2012; Nishie et al., 2012).

Lantibiotics are bacteriocins synthesized by lactic acid bacteria (LAB). These bacteriocins exhibit a cationic and amphiphilic structure with a low molecular weight, from ca. 3 kDa to 10 kDa, and are subdivided in classes I and II. Class I lantibiotics are formed by small peptides containing 19–38 aminoacid moieties and contain unusual modified aminoacids, such as lanthionine and 3-methyllanthionine and some dehydrated aminoacids. These aminoacids are introduced via enzymatic modification following the translation process, conferring to lantibiotics stability against heat, to a wide range of pH, against proteolysis and resistance to oxidation (Rajaram et al., 2010; Lee and Kim, 2011; Nishant et al., 2011; Lohans and Vederas, 2012; Nishie et al., 2012).

Lantibiotics can also be classified according to their peptide maturation pathway, being classified as type A lantibiotics (I) (which can be modified by enzymes LanB and LanC) and class II, which are formed by types A (II) and B, modified by enzyme LanM (Rajaram et al., 2010; Lee and Kim, 2011; Nishie et al., 2012).

Class II bacteriocins (see Fig. 10), unlike lantibiotics, are not modified enzymatically after their translation processes, so they do not possess unusual modified aminoacid sequences and, due to their heterogeneous nature, classification of these bacteriocins becomes even more complex, leading to several grouping approaches (Jeevaratnam et al., 2005; Rajaram et al., 2010; Nishant et al., 2011; Cui et al., 2012; Lohans and Vederas, 2012; Nishie et al., 2012; Kawada-Matsuo et al., 2013). Bacteriocins from Class II are divided into four groups: (i) class IIa, including pediocins (bacteriocins active against *Listeria monocytogenes* and having the N-terminal region YGNGVXC (a characteristic of this class), synthetized by *Lactobacillus plantarum* and *Pediococcus sp*); (ii) class IIb, including two-peptide bacteriocins (these two peptides are not modified by enzymatic activity and are responsible for the bacteriocin activity), examples of which are ABP-118, G and Q lactococcins; (iii) class IIc, made up of bacteriocins formed by cyclic peptides containing an N and C-terminal covalently linked by a peptide bond, with reduced content in cysteine residues, essential to ensure the antibacterial activity (examples in this class include gassericin A, uberolysin and lactocyclicin Q); (iv) class IId, broadly diversified because it comprises the remaining bacteriocins that do not fit on the other classes (examples in this class include divergicin A and the "leaderless bacteriocins" as aureocin A70 and enterocin L50 that are synthetized without an N-terminal sequence) (Cotter et al., 2005; Jeevaratnam et al., 2005; Zendo et al., 2006; Nissen-Meyer et al., 2009; Papagianni and Anastasiadou, 2009; Rajaram et al., 2010; Lee and Kim, 2011; Nishant et al., 2011; Cui et al., 2012; Nishie et al., 2012).

Class III bacteriocins includes those composed by thermosensitive proteins of high molecular weight (higher than 30 kDa), which have a different mechanism of action than the other bacteriocins. Due to their lytic character, this type of bacteriocins is now called bacteriolysins. Finally, class IV bacteriocins comprises a complex group of proteins associated with lipids and carbohydrates, which are essential to their activity (Cotter et al., 2005; Jeevaratnam et al., 2005; Papagianni and Anastasiadou, 2009; Rajaram et al., 2010; Lee and Kim, 2011; Nishant et al., 2011; Lohans and Vederas, 2012).

5.1. Structure and mechanism of action of bacteriocins

Bacteriocins are generally constituted by a C-terminal domain, responsible for pore formation in the bacterial cell membrane, that is connected to a N-terminal domain, responsible for binding the bacteriocin to a receptor present in the bacterial cell surface. Initially, the N-terminal domain has a leading peptide that renders the bacteriocin inactive and prevents it from acting against its own producing bacteria. This leading peptide is subsequently cleaved by enzymes, such as protease LanP in lantibiotics, or LanT in class II bacteriocins, activating the bacteriocin in the intracellular environment. Subsequently, the bacteriocin is transported to the extracellular environment by specific transporter proteins (Cotter et al., 2005; Oppegard et al., 2007; Nishie et al., 2012).

Due to their structural diversity, bacteriocins exhibit different mechanisms of action and, in many cases, only one type of bacteriocin exhibits more than one type of mechanism of action to attack the target bacteria. Additionally, bacteriocins can act in dif-

Table 4
Classification, characteristics and examples of bacteriocins.

Bacteriocin classification		Characteristics	Examples
Class I (Lantibiotics)	Lantibiotics I	Unusual aminoacids introduced by LanB and LanC	Nisin A, Subtilin, Epidermin, Pep5
	Lantibiotics II	Unusual aminoacids introduced by LanM	Lacticin 481, Nukacin ISK-1, Mersacidin, Lacticin 3147
Class II	Class IIa	Pediocin-like bacteriocins, specific against *Listeria monocytogenes*	Pediocin PA-1&/AcH, Leucocin A
	Class IIb	Two-peptide bacteriocins	Lactococcin G, Lactococcin Q
	Class IIc	Cyclic bacteriocins	Enterocin AS-48, Gassericin A, Lactocyclicin Q
	Class IId	Linear, non-pediocin-like, one-peptide bacteriocins	Lactococcin A, Lacticin Q
Class III	–	Heat labile proteins of large molecular weights	–
Class IV	–	Glycoproteins or lipoproteins that require non-protein moieties for their activity	–

Source: adapted from Jeevaratnam et al. (2005), Nishant et al. (2011), Bodaszewska-Lubas et al. (2012) and Nishie et al. (2012).

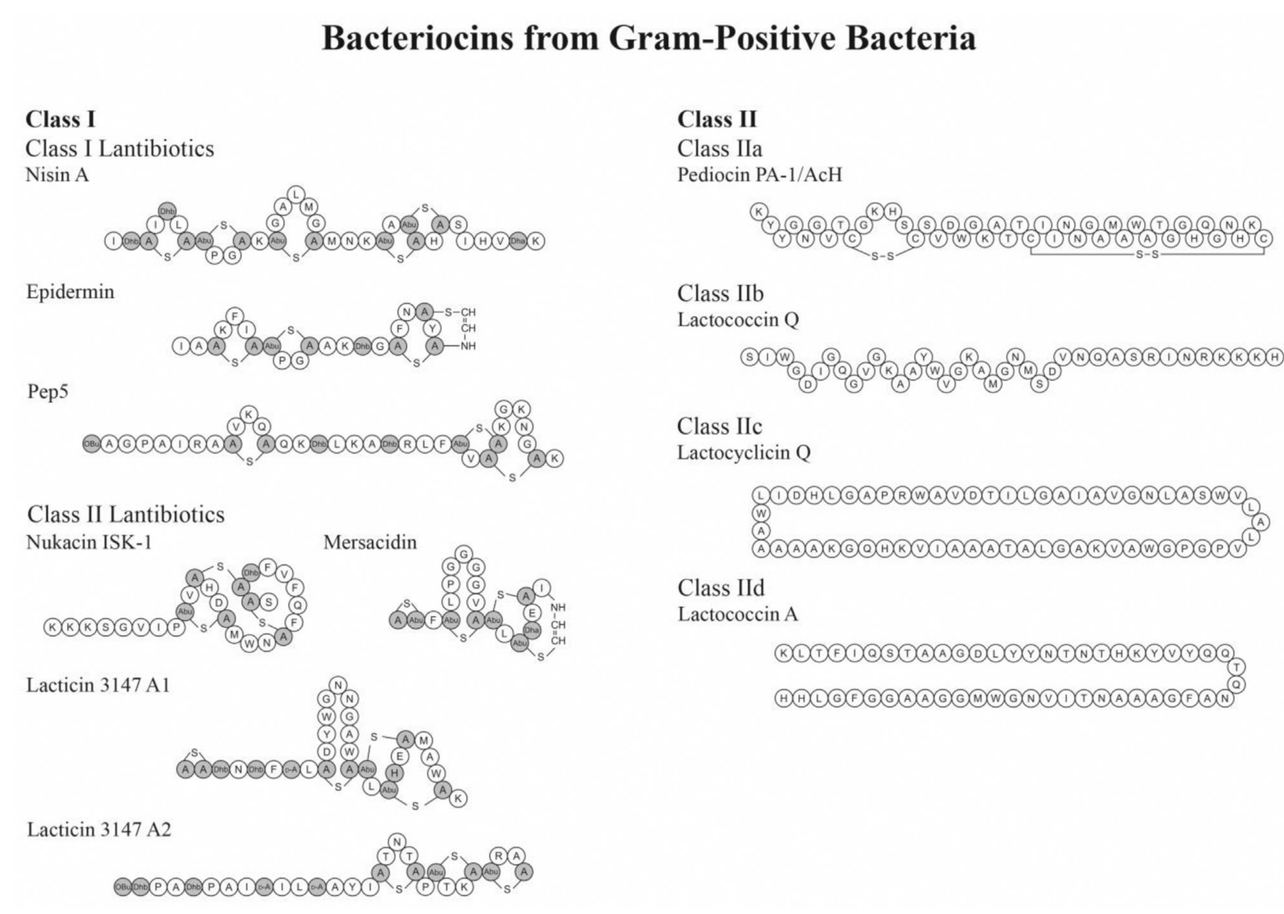

Fig. 10. Primary structure of several Class I and II bacteriocins.

Source: adapted from Nishie et al. (2012).

ferent cell functions, such as transcription, translation, replication and cell membrane processes (Jeevaratnam et al., 2005; Lee and Kim, 2011). Most bacteriocins act via pore formation in bacterial cell membranes, affecting their integrity and leading to the collapse of the phospholipid bilayer, consequently causing cell death (Jeevaratnam et al., 2005; Nishie et al., 2012).

In the case of class I lantibiotics, such as nisin A, because these are cationic, they electrostatically bind to the bacterial phospholipid membranes, due to their anionic character, which favors the interaction between the cellular membrane and the N-terminal domain of the bacteriocin. This domain establishes a link with pyrophosphate II in the lipid cell membrane, inhibiting the pep-

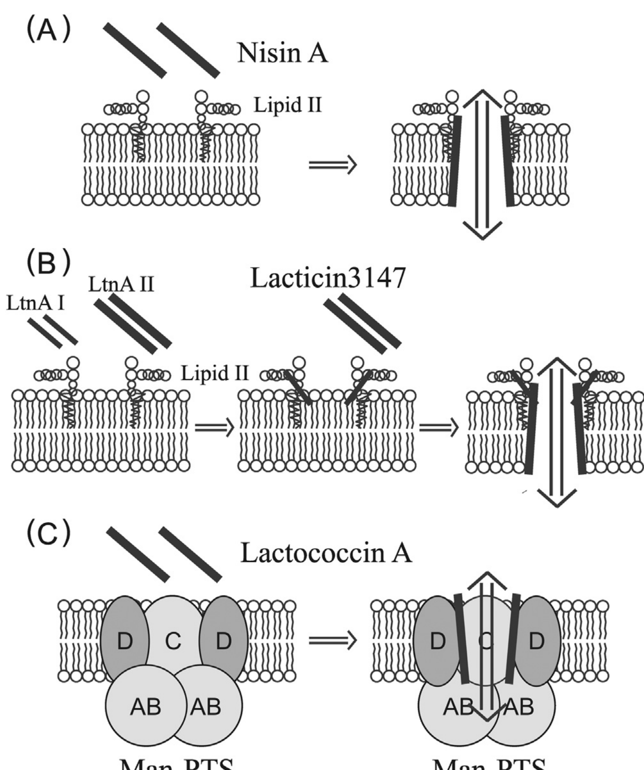

Fig. 11. Mechanisms of action of classes I and II bacteriocins; nisin A (A), lacticin 3147 (B) and lactococcin A (C).
Source: adapted from Nishie et al. (2012).

tidoglycan synthesis, increasing cell permeability and thus leading to the formation of pores in the membrane, triggering an efflux of calcium and magnesium ions. These cations neutralize the anionic charge of phospholipids and some low molecular weight intracellular molecules, triggering a collapse of the phospholipid bilayer followed by cell death.

Due to the high specificity of nisin A to lipid II, it can promote therapeutic action at minimal amounts (in the order of nanograms). Is important to mention that mutations in the N-terminal domain may inactivate all this process, because this is a vital region for the AMP (see Fig. 11A) (Fernández et al., 2008; Lee and Kim, 2011; Nishie et al., 2012; Kawada-Matsuo et al., 2013).

In the case of the bacteriocin lacticin 3147, which has a dipeptide structure and a mechanism of action different from the ones mentioned above, it is constituted by the peptides LtnA1 and LtnA2 that act synergistically. LtnA1 is responsible for recognizing and interacting with lipid II present in the bacterial cell membrane, forming a dimeric complex which binds to LtnA2 yielding a trimeric complex, capable of inhibiting the peptidoglycan synthesis and promoting the formation of pores in the bacterial cell membrane. Subsequently, the pores formed lead to extensive ion and ATP losses, killing the bacteria (see Fig. 11B) (Nishie et al., 2012).

Class IIa bacteriocins, such as pediocin PA-1/AcH, promote cell death also via pore formation in the bacterial cell membrane, but by a different mechanism from those that have been mentioned before. Since these bacteriocins are cationic, they initially promote the electrostatic attraction to the cell membrane (negative in character) which allows them to recognize the mannose phosphotransferase system or Man-PTS (a protein complex involved in the transport of carbohydrates, present on the surface of the cell membrane of bacteria susceptible to this bacteriocin) as receptor. Then, its amphiphilic C-terminal domain in the shape of a α-helix is inserted into the cell membrane, inducing the formation of hydrophilic pores, affecting the membrane proton-motive force, causing a depletion of ATP with consequent cell death (Nissen-Meyer et al., 2009; Kjos et al., 2010; Cui et al., 2012; Lohans and Vederas, 2012; Nishie et al., 2012).

Class IIb bacteriocins includes, for example, lactococcin G (Gα peptide and Gβ peptide) and lactococcin Q (Qα peptide and Qβ peptide) synthetized by *Lactococcus lactis*. Both bacteriocins are composed of two different peptides that act synergistically in an equimolar proportion, to ensure antibacterial activity. This class of bacteriocins increases the permeability of the cell membrane, promoting a depletion of cations essential for cell viability, such as calcium and magnesium and phosphates, and a reduction in the levels of ATP, causing cell death (Zendo et al., 2006; Nishie et al., 2012).

Class IIc bacteriocins exhibit a cyclic structure, and their N- and C-terminal domains are covalently bound. However, despite several structural differences in relation to class IIb bacteriocins, both display the same mechanism of action. Some class IIc bacteriocins, such as enterocin AS-48, gassericin A, subtilosin A and carnocyclin A, may exert their antimicrobial mechanism of action without the presence of receptors on the bacterial cell membrane (Nissen-Meyer et al., 2009; Nishie et al., 2012).

Class IId bacteriocins exhibit several different structures and, consequently, different mechanisms of action. However, lactococcins A and B display the same mechanism of action as class IIa bacteriocin pediocin PA-1, but pediocin only requires the IIC portion of Man-PTS to have activity, and lactococcins A and B require the portion IIC and IID to recognize as receptor and start their activity (see Fig. 11C) (Nissen-Meyer et al., 2009; Nishie et al., 2012).

5.2. Bacterial resistance to bacteriocins and bacteriocin toxicity

There are already numerous reports in the literature regarding bacterial resistance to bacteriocins, and some studies show that the mechanism of bacterial resistance to nisin A and other class IIa bacteriocins is connected either to an increase of membrane fluidity, which reduces the efficacy of bacteriocin insertion on the cellular membrane, or to a neutralization of cell-surface charge, which increases the ability of the bacteria to repel the cationic AMPs. These mechanisms of bacterial resistance may be associated with mutations in bacterial DNA (Lohans and Vederas, 2012; Nishie et al., 2012).

As an example, a mutation in the sigma factor B, or SigB, a mediator involved in the response of bacteria to adverse environmental conditions, is responsible for making *Listeria monocytogenes* (an etiological agent responsible for triggering listeriosis, an opportunistic infection that mainly affects immunocompromised individuals and pregnant women) tolerant to nisin A and lacticin 3147 (Begley et al., 2006; Lohans and Vederas, 2012; Nishie et al., 2012).

The VirRS system regulates the expression of operons *dltA* (the products of this operon neutralize, by esterification with D-alanine, the polyanionic polymers of teichoic acid of Gram-positive bacteria, which increases the ability of these bacteria to repel cationic AMPs) and *mprF* (a large membrane protein present in Gram-negative and Gram-positive bacteria, that protects some of these bacteria against AMPs by reducing the negative charge of the cell membrane via addition of the amino acid L-lysin to phosphotidylglycerol, causing resistance to cationic AMPs) in *Listeria monocytogenes*, and is responsible for reducing the susceptibility of this bacteria to cationic AMPs. Nevertheless, this bacterial defense system can be inhibited by D-alanylacyl-sulfamoyl-adenosine, which blocks the D-alanyl ligase, preventing the neutralization of the bacterial cell membrane and consequently increasing the susceptibility of Gram-positive bacteria, such as *Listeria monocytogenes* and *Bacillus subtilis*, to cationic AMPs including nisin A (May et al., 2005; Lohans and Vederas, 2012; Nishie et al., 2012).

However, the mechanisms of bacterial resistance to class IIa bacteriocins are correlated with two resistance mechanisms at the same time, involving the Man-PTS of *Listeria monocytogenes* and *Lactococcus lactis*. In the first resistance mechanism, due to a mutation, a down regulation of Man-PTS gene expression occurs leading to a decrease in the number of receptors on the bacterial cell membrane, causing resistance to this class of bacteriocins. The second resistance mechanism involves a normal expression of the Man-PTS gene, both on resistant and susceptible bacteria, suggesting that the resistance to this class of bacteriocins is correlated with variations on the composition of the cell membrane surface (Gravesen et al., 2002; Vadyvaloo et al., 2002, 2004; Tessema et al., 2009; Kjos et al., 2011; Lohans and Vederas, 2012).

The bacterial resistance to class I bacteriocins is strongly associated with the system *graRS*, *braRS* and *vraSR*, which allows *Staphylococcus aureus* to easily adapt to the new environmental conditions and become resistant to bacteriocins such as nisin A and nukacin ISK-1 (Kawada-Matsuo et al., 2013).

5.3. Application of bacteriocins

5.3.1. Application of bacteriocins as biopreservatives

Bacteriocins such as nisin and pediocin PA-1 are widely used in the food industry because they inhibit the growth of foodborne pathogenic and spoilage-causing bacteria, such as *Clostridium botulinum*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, among others (Settanni and Corsetti, 2008; Lohans and Vederas, 2012; Nishie et al., 2012; Huang et al., 2013).

The most applied bacteriocins in the food industry as biopreservatives are the LAB-derived bacteriocins, synthetized by lactic acid bacteria (LAB). These bacteria can be found in products such as cheese, yoghurt and other fermented dairy products. Currently, lantibiotics were granted the GRAS (Generally Regarded As Safe) status by FDA, because they are peptides and exist in the gastrointestinal tract (GIT), and so they are easily metabolized to aminoacids by digestive proteases.

It is noteworthy that bacteriocins produced by LAB have no taste or odor, show a high stability and exhibit reduced and highly specific spectrum of action for a particular bacterium, which is highly interesting for their applications in the food industry (Jeevaratnam et al., 2005; De Vuyst and Leroy, 2007; Nissen-Meyer et al., 2009; Papagianni and Anastasiadou, 2009; Rajaram et al., 2010; Lee and Kim, 2011; Lohans and Vederas, 2012; Nishie et al., 2012).

Certain particular species of LAB have properties that allows them to be used in the treatment of GIT infections caused by *Helicobacter pylori*, *Escherichia coli* and *Salmonella* (Jeevaratnam et al., 2005; De Vuyst and Leroy, 2007; Duquesne et al., 2007; Nishie et al., 2012).

Another bacteriocin, enterocin RM6, was isolated from *Enterococcus faecalis* present in raw milk. This bacteriocin was purified via reverse-phase HPLC and proved to be active against Gram-positive bacteria including *Listeria monocytogenes*, *Bacillus cereus* and MRSA strains, preventing the proliferation of these pathogenic bacteria in food, suggesting that this bacteriocin can be widely applied in the food industry as biopreservative (Huang et al., 2013).

5.3.2. Application of bacteriocins as therapeutic agents

In addition to being extensively applied in the food industry as biopreservatives, some lantibiotics such as nisins A and F, mersacidin, mutacin 1140, lactacin 3147, and pediocin AcH/PA-1, have proved to be active against MRSA and vancomycin resistant enterococci (VRE) strains, establishing themselves as a potential therapeutic strategy to fight multidrug-resistant bacteria and bacterial infections (Sang and Blecha, 2008; Papagianni and Anastasiadou, 2009; Collins et al., 2010; Bodaszewska-Lubas et al., 2012; Lohans and Vederas, 2012; Nishie et al., 2012).

Recently, a scientific study has revealed that, when injected into infected mice with *Staphylococcus aureus*, nisin F inhibited the growth of this bacteria during a timeframe of at least 15 min (Brand et al., 2010). Nisin A was also demonstrated to be an efficient alternative to antibiotics in the treatment of mastitis caused by *Staphylococcus aureus* (Fernández et al., 2008). In the study by Fernández et al. (2008), eight women with mastitis caused by *Staphylococcus aureus* were randomly divided into two groups, the first group of which was administrated with a solution containing 6 μg/mL of nisin A, on the nipple and areola, for a period of two weeks. In the second group, the control one, an identical solution to the previous one was also administered, but lacking nisin A. According to this study, on day 0, the number of bacteria (CFU/mL) present in the breast milk of the women in both groups were similar. However, after 14 days of treatment, the group that received the solution containing nisin A showed a significant reduction in the number of bacteria in their breast milk and these women became free from signs of infection (Jeevaratnam et al., 2005; Fernández et al., 2008; Nishie et al., 2012). Furthermore, according to Kruszewska et al. (2004), mersacidin synthesized by *Bacillus* sp. is active against several strains of MRSA, eliminating these bacteria that colonized the nasal mucosa of mice. Another study performed *in vivo* with pediocin PA-1, administered orally, revealed that this bacteriocin did not disturb the GIT microflora of the mice under study, but the same did not happen when using conventional chemical antibiotics, such as penicillin and tetracycline (Le Blay et al., 2007).

Bacteriocins are peptidic structures and their route of administration may be conditioned since they are immunogenic agents and can trigger an immune response. Nevertheless, a study undertaken in rats where pediocin AcH was administered intraperitoneally led to the observation that no antibodies were produced against this bacteriocin, strongly suggesting that, apparently, this bacteriocin is non-immunogenic and free from side effects (Lohans and Vederas, 2012).

According to two other studies, also developed in mice, an intravenous administration of piscicolin 126 and diversin RV41 (both class IIa bacteriocins) proved to be effective 30 min after infection of mice with *Listeria monocytogenes*, allowing to conclude that this class of bacteriocins also demonstrates therapeutic activity (Ramaswamy et al., 2007; Bernbom et al., 2009; Lohans and Vederas, 2012).

A bacteriocin produced by *Brevibacillus* sp., IM-9, called laterosporulin, purified by reverse-phase HPLC, produced a molecular weight of 5.6 kDa, and displays a N-terminal sequence stable to high temperatures (up to 120 °C), tolerant to pH variations (between 2 and 10) and a high stability against proteases, without affecting its antibacterial activity. Additionally, laterosporulin exhibits a broad spectrum of action, being active against both Gram-negative and Gram-positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes* and *Pseudomonas aeruginosa*. This bacteriocin has a MIC of 500 μg/mL and leads to changes in the shape and morphology of *Escherichia coli* after 4 h of exposure, accumulation of cellular debris and lysed cells in the preparation. Therefore, laterosporulin is an innovative broad-spectrum bacteriocin, with potential applications in the treatment of several bacterial infections (Singh et al., 2012).

In spite of the fact that bacteriocins appear to be an outstanding therapeutic alternative to chemical antibiotics, the pharmaceutical industry remains reluctant to fund both research in this area and production of preparations containing bacteriocins, mainly due to (i) the low yield obtained from fermentative processes; (ii) production of unstable products; (iii) the excessively expensive and time consuming (downstream) purification processes; and (iv) legislation problems associated with these products.

Nowadays, bacteriocins can be genetically engineered in order to increase their potency, stability, and spectrum of action, adjust-

Table 5
Examples of bacteriocins produced by bacteria isolated from the marine environment.

Producing bacteria	Origin of isolate	Bacteriocin	Bacteria inhibited
Listonella anguillarum AVP10	Healthy and infected catfishes (*Arius thalassimus*)	Vibriocin AVP10	*Escherichia coli* and *Listonella anguillarum* AVS9
Vibrio vulnificus	Water samples from Wilmington (NC, USA)	1W1, BC1, BC2	*Vibrio vulnificus*, *Vibrio cholera*, *Vibrio parahaemolyticus*, *Plesiomonas shigelloides* and *Escherichia coli*
Aeromonas hydrophila	Water tank containing alligators	BLIS	*Staphylococcus aureus*
Pseudoalteromonas Species Strain X153	Substrates on the littoral of Brittany	Antibiotic protein P-153	Ichthyopathogenic *Vibrio*, *Staphylococcus epidermidis*, *Propionibacterium acnes* and *Propionibacterium granulosum*
Vibrio sp. Strain NM 10	Spotnape ponyfish *(Leiognathus nuchalis)* intestine	BLIS	*Pasteurella piscicida* K-III, *Escherichia coli*, *Enterococcus seriolicida* and *Vibrio vulnificus*

Source: adapted from Desriac et al. (2010).

ing their application to many different pathological situations and improving their applications *in vivo*. For example, using genetic engineering approaches, it has been introduced an additional lysin moiety at the N-terminal domain of sakacin 44 K and, in sakacin T20 K, a neutral moiety has been replaced by a cationic one, leading to an increase in the net positive charge of both bacteriocins, promoting a consistent electrostatic interaction between the net positive charge of the mutant bacteriocins and the negative charge of the bacterial cell membrane, and thereby leading to an increase in the potency and therapeutic activity of these GEB's (genetically engineered bacteriocins) (Kazazic et al., 2002). Additionally, the replacement of a methionine moiety by a hydrophobic one, was shown to reduce the susceptibility of pediocins and two-peptide bacteriocins to oxidation phenomena, responsible for a dramatic reduction in their antibacterial activity (Nissen-Meyer et al., 2009; Lohans and Vederas, 2012; Nishie et al., 2012).

Presently, researchers are trying to develop chimeric bacteriocins by swapping the N-terminal domain of a certain class of bacteriocins with a C-terminal domain of another class, which may lead to the formation of more powerful and active bacteriocins, as it happened with pediocin PA-1, which led to the development of several chimeric pediocins that exhibited an activity equal or higher than that of the original one (Tominaga and Hatakeyama, 2007; Lohans and Vederas, 2012).

Interestingly, over the past few years, several bacteriocins were isolated from marine environment, from a countless number of bacteria-producing bacteriocins, such as *e.g. Vibrio*, *Pseudoalteromonas*, *Aeromonas*, *Alteromonas*, among others (see Table 5). Thus, due both to the high biodiversity of this ecosystem and to the fact that it still is underexplored, it is likely that in the near future countless bacteriocins may be discovered, with a higher antibacterial activity and a wider spectrum of action, providing a new arsenal against multidrug-resistant bacteria to the community, definitely establishing bacteriocins as an attractive strategy and feasible alternative to antibiotics (Desriac et al., 2010).

5.3.3. Application of bacteriocins produced by probiotic bacteria

Probiotics are live microorganisms which, when consumed in appropriate concentrations, exert a beneficial effect on the host's health, at a prophylactic level, helping to restore or maintain the normal gut microflora (Gillor et al., 2008; Bodaszewska-Lubas et al., 2012; Lohans and Vederas, 2012). However, according to Dabour et al. (2009), purified bacteriocins exhibit a higher therapeutic activity when there is an established infection, which was demonstrated by the administration of pediocin PA-1 and *Pediococcus acidilactici* UL5 (a bacterium that synthetizes pediocin PA-1) in mice infected with *Listeria monocytogenes*.

Over the years, probiotic bacteria has revealed an effective antimicrobial activity by preventing the growth of pathogenic bacteria in the gut, and they can be used in the treatment of bacterial infections of the GIT and vaginal mucosa. Probiotic bacteria may be applied in the GIT, producing *in situ* a variety of bacteriocins, which contribute to the maintenance of a proper balance between the host and the intestinal microflora, preventing the proliferation of pathogenic microorganisms and helping in digestion. There are many bacteria that are applied as probiotics in the GIT due to bacteriocin production, such as *Lactobacillus salivarius* UCC118 (responsible for the production of bacteriocin Abp118, that inhibits the growth of *Listeria monocytogenes*), *Lactobacillus casei* L26 (which inhibits the growth of *Escherichia coli* 0111 (enterohemorrhagic) and *Listeria monocytogenes*), *Lactobacillus johnsonii* LA1 and *Lactobacillus acidophilus* LB (which inhibits the growth of *Helicobacter pilori*), and *Enterococcus mundtii* ST4SA (producer of ST4SA bacteriocin, which is active against both Gram-positive (*Enterococcus faecalis*, *Streptococcus pneumoniae* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*)) (Corr et al., 2007; De Vuyst and Leroy, 2007; Gillor et al., 2008; Gotteland et al., 2008; Granger et al., 2008; Nishant et al., 2011).

In addition, bacteriocins produced by probiotic bacteria can be applied in the oral cavity, reducing the number of etiologic agents responsible for the proliferation of dental caries, such as *Streptococcus salivarius* and *Streptococcus mutans*, by using a strain of *Streptococcus mutans* A2JM (genetically engineered) which produces mutacin 1140 involved in the prevention of dental caries (Hillman et al., 2007). It is important to mention that *Streptococcus salivarius* K12 synthetizes two lantibiotics, termed salivaricin A and B, that inhibit the proliferation of *Streptococcus pyogenes*, a saprophytic bacterium responsible for respiratory tract infections such as pharyngitis in immunocompromised patients. In the case of salivaricin B, it allows to combat halitosis caused by *Prevotella* spp., *Eubacterium saburreum* and *Micromonas micros* (Burton et al., 2006a,b; Gillor et al., 2008). There is already available on the market BLIS K12® and BLIS M18™ in the form of lozenges, containing *Streptococcus salivarius* K12 and *Streptococcus salivarius* M18 that produce bacteriocins, which restore the normal microflora of the oral cavity, contributing to the reduction of halitosis and preventing dental caries (Burton et al., 2013). Bacteriocins produced

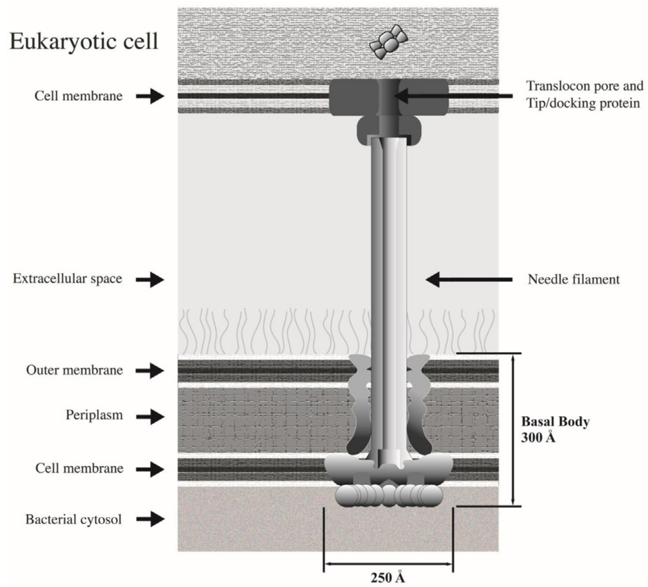

Fig. 12. Bacterial organelle, with a structure resembling a needle that secrets toxins directly to the intracellular environment of the host.
Source: adapted from Marlovits and Stebbins (2010).

by probiotic bacteria can also be applied in the vaginal mucosa, promoting the replacement and maintenance of the natural flora while preventing the spread of pathogenic bacteria that trigger bacterial vaginosis. Examples of this include vaginal applications of *Lactobacillus jensenii* 5L08 (produces a bacteriocin active against *Gardnerella vaginalis*, *Candida albicans* and *Escherichia coli*), *Lactobacillus salivarius* CRL 1328 (binds to the epithelial cells of the vaginal mucosa and prevents the attachment of pathogenic bacteria while inhibiting the growth of *Enterococcus faecalis*, *Enterococcus faecium*, *Neisseria gonorrhoeae* and *Staphylococcus aureus*), and *Lactobacillus fermentum* HV6b MTCC10770 (present in the vaginal flora, produces fermenticin HV6b and inhibits the growth of *Bacteroides* sp., *Gardnerella vaginalis*, *Mobiluncus* sp., *Staphylococcus* sp. and *Streptococcus* sp., acts as an antineoplastic agent, and can also be applied in contraceptive products) (Kaewsrichan et al., 2006; Gillor et al., 2008; Kaur et al., 2013).

6. Other potential alternatives

6.1. Antivirulence strategies

Most chemical antibiotics interfere with the cellular processes. However, there are innovative alternatives under development that interact with virulence factors, which are primarily responsible for triggering infections, making it easier for the immune system to fight them, *e.g.* (i) inhibition of toxin secretion – most Gram-negative bacteria release toxins via their type III secretion system (T3SS), which involves the formation of a bacterial organelle with a structure like a needle that secrets toxins directly into the intracellular environment of the host (see Fig. 12), as is the case with INP0403 (a salicylidene acylhydrazide-mediated inhibition of type III secretion system used in the treatment of infections caused by *Mycobacterium tuberculosis* and in the treatment of tuberculosis, reducing the transcription of genes involved in this mechanism); (ii) use of specific antibodies that inhibit the toxins produced by *e.g. Clostridium botulinum*, via antibodies H3H, F3A and F4H, that suppress the catalytic domain of neurotoxin serotype A; (iii) interference with the quorum-sensing of bacteria via 5′-methylthio-DADMe-ImmucillinAs, 5′-ethylthio-DADMe-ImmucillinAs and 5′-butylthio-DADMe-ImmucillinAs, which inhibit the 5′-Methylthioadenosine nucleosidase (MTAN), an enzyme involved in quorum-sensing of *Escherichia coli* and *Vibrio cholerae*, reducing the biosynthesis of autoinducers AI-1 and AI-2 (signaling molecules), the ability to form biofilms, reducing the infection capacity and the resistance to antibiotics; and (iv) use of inhibitors of the pili biosynthesis, or *pilicides*, to reduce the adhesion of bacteria to the epithelium and consequently reduce biofilm formation (Galan and Wolf-Watz, 2006; Hudson et al., 2007; Kaufmann et al., 2008; Gutierrez et al., 2009; Layton et al., 2010; Marlovits and Stebbins, 2010; Pang et al., 2010; Fernebro, 2011).

These antivirulence strategies have as main advantage the fact of being specific to virulence factors that only exists in pathogenic bacteria, so they do not affect the commensal flora in the host. In addition, these antibacterial approaches can be administered either topically or systemically, and can be used as prophylaxis in cases of bioterrorism and epidemics (Moayeri et al., 2006; Fernebro, 2011).

6.2. Anti-bacterial antibodies

Treatment with antibacterial antibodies is not yet a reality, since most antibodies already discovered are still in clinical trials, as illustrated by the data in Table 6. However, this type of therapy is widely applied in the treatment of cancer (Saylor et al., 2009; Oleksiewicz et al., 2012).

Regarding the advantages of antibodies, this alternative therapy may be used in the near future in the prevention and treatment of bacterial infections in animals, without affecting their commensal flora. As antibodies are extremely selective, they are extremely safe and effective. However, this therapy shows some disadvantages as well, such as: (i) the synthesis and purification processes are expensive and, as they have a very small market, the final product is very expensive; (ii) they can only be administered systemically; and (iii) they may lose their effectiveness over time, due to the antigenic variation of the bacteria (Bebbington and Yarranton, 2008; Fernebro, 2011).

Table 6
Examples of antibacterial antibodies currently under clinical trials.

Antibody	Target bacteria	Producing Company
Anthim (ETI-204)	*Bacillus anthracis*	Elusys Therapeutics
Raxibacumab	*Bacillus anthracis*	Human Genome Sciences
Valortim (MDX-1303)	*Bacillus anthracis*	PharmAthene/Medarex
CDA1/CDB1	*Clostridium difficile*	Medarex/MassBiologics/Merck
ShigamAbs	Shiga toxin-producing *Escherichia coli*	Thallion Pharmaceuticals
Urtoxazumab	Shiga toxin-producing *Escherichia coli*	Teijin
Anti-Pseudomonas IgY	*Pseudomonas aeruginosa*	Immunsystem
KB001	*Pseudomonas aeruginosa*	KaloBios Pharmaceuticals/Sanofi Pasteur
Panobacumab (KBPA101)	*Pseudomonas aeruginosa*	Kenta Biotech
Pagibaximab	*Staphylococcus aureus*	Biosynexus

Source: adapted from Fernebro (2011).

Recently, a phase I clinical trial was conducted in infants, using pagibaximab (BSYX-A110), an anti-LTA human chimeric IgG1 antibody, developed via recombinant DNA technology. In this clinical trial, BSYX-A110 was administered systemically with three infusions, containing from 60 to 90 mg/kg Pagibaximab. This antibody is specific to the lipoteichoic acids of *Staphylococcus aureus*, which are essential to its viability. When it binds to the lipoteichoic acids, this antibody stimulates phagocytosis and inhibits the release of cytokines, responsible by the induction of organ failure. In this clinical trial, Pagibaximab was considered well tolerated, safe and effective in the treatment and prevention of infections caused by *Staphylococcus aureus* (Bebbington and Yarranton, 2008; Weisman et al., 2011).

Presently, 2E9IgA1, an antibody specific for *Mycobacterium tuberculosis*, was administered intranasally associated with IFN-γ, reducing significantly the respiratory infection in transgenic mice with human CD89 receptor. This clearly indicates that 2E9IgA1 has a high affinity for the α-crystalline receptor (antigen) of *Mycobacterium tuberculosis* and for the CD89 receptor (FcαRI) present in macrophages and neutrophils. According to this study, developed by Balu and colleagues (2011), it is believed that 2E9IgA1 can act in two ways, either by binding to the CD89 receptor of alveolar macrophages, which stimulates the phagocytosis of the bacteria, or by binding to CD89 receptor of neutrophils that subsequently exert a bactericidal effect. This antibody may contribute, in the near future, for reducing the time of chemotherapy in patients with tuberculosis, for reducing tuberculosis transmission and for reducing the development of new multi-drug resistant strains (Balu et al., 2011).

6.2.1. Radioimmunotherapy

Radioimmunotherapy (RIT) is a technique that has been used in the treatment of bacterial infections caused by *Streptococcus pneumonia*, using the monoclonal antibody D11, which specifically binds to PPS8, a polysaccharide present on the bacteria capsule. D11 was associated with the radioactive isotope ^{213}Bi, administered at a concentration of 2.96 GBq (equivalent to 80 μCi) and allowed survival of 87% to 100% of the test population. In addition, this study demonstrated that after 3–14 days of initiating the treatment, the animals did not show any signs of the pathology (Dadachova et al., 2004).

In another study using RIT, it was demonstrated that RIT can also be used to treat infections caused by toxin-producing bacteria, such as *Bacillus anthracis*, by using the radioactive complexes [^{213}Bi]10F4γ1 and [^{213}Bi]14FAγ2b, which exhibited bactericidal action since toxins accumulate in the periphery of this bacterium, thus becoming an easy target (Rivera et al., 2009; Saylor et al., 2009; Nosanchuk and Dadachova, 2012).

6.3. Vaccines

Currently, there are new novel methods to identify antigens. Genetic engineering and bioinformatics allows the identification and synthesis of epitopes responsible for triggering the immune response, leading to the development of recombinant vaccines. Such vaccines are composed of (at least) one modified antigen which, when administered with adjuvants or plasmids, induce an immune response against the pathogen. In addition, antimicrobial vaccines may contain live recombinant microorganisms such as *Mycobacterium bovis*, *Listeria monocytogenes*, *Salmonellae* spp. and *Shigellae* spp., the genome of which was inserted with a gene that encodes for a desired antigen, thus functioning as a vector.

Some DNA vaccines already exist in the market. In producing such vaccines, genes coding for numerous antigens were inserted into a plasmid and, when they are administered intramuscularly, can induce the production of specific antibodies against several pathogens (Fernebro, 2011; Nascimento and Leite, 2012; Nabel, 2013).

Vaccines currently under clinical trials include (i) vaccine IC43, applied prophylactically against *Pseudomonas aeruginosa* (this vaccine is composed of two recombinant antigens that are present on the bacterium surface); (ii) vaccines GSK2392105A and SA3Ag that contain 3–4 antigens, respectively, inducing immunization against *Staphylococcus aureus*; (iii) vaccines containing vesicles from the outer membrane of bacteria, protecting against *Neisseria meningitidis*, *Burkholderia pseudomallei* and *Escherichia coli*; (iv) a vaccine containing a genetically inactivated α-toxin (HlaH35L), which contributed to immunization of mice when infected by *Staphylococcus aureus* (Doring and Pier, 2008; Nieves et al., 2011; Brady et al., 2013; Kim et al., 2013).

Countries that adopted anti-pneumococcal vaccination, as the United Kingdom, have seen a reduction in the incidence of pneumococcal infections by ca. 41%. The reduction in the appearance of infections by *S. penumoniae* such as otitis, pharyngitis and sinusitis, leads to a reduction in the number of doctor visits, antibiotic prescription and, consequently, to a decrease in the appearance of antimicrobial resistance (Gladstone et al., 2011).

7. New sources for new potentially antimicrobial molecules

During the years of 1960 and 1970, search and discovery for new antimicrobial agents derived from soil microorganisms was quite intensive, however it came to an end quite quickly due to the difficulties in growing in the lab the vast majority of microbial species found in the nature (Lewis, 2012). Quite recently, Nichols et al. (2010) developed a system called iChip, which allows to grow soil microorganisms that were ungrowable in the laboratory until then. Such system is composed of hundreds of diffusion chambers at a nanoscale, each one of them inoculated with a single microbial cell, resulting in "*in situ*" monospecific cultures. Such system allows to grow, identify and, subsequently, evaluate the antimicrobial activities of microorganisms ungrowable in the laboratory. Such a strategy appears highly promising since ca. 99% of all microbial species in the nature could not, until then, be grown in the laboratory (Lewis, 2013). The first results of this new approach already started to appear. Very recently, Ling et al. (2015), using such system, isolated a new substance, teixobactin, from growing a new species of β-proteobacteria, Gram-negative, temporarily and provisionally named *Eleftheria terrae*. The new molecule (teixobactin) exhibited a high activity against Gram-positive microorganisms and also activity against *Mycobacterium tuberculosis*, *Clostridium difficile* and *Bacillus anthracis*. Teixobactin appears to have as mechanism of action, inhibition of the synthesis of bacterial cell wall in previous steps, such as vancomycin and teicoplanin. Its antibacterial activity was effective at very low concentrations: *Staphylococcus aureus* strains, both MRSA and MSSA, were sensible at concentrations of 0.25 μg/mL. Regarding *Enterococcus faecium* (VRE) and *Enterococcus faecalis* (VRE), its action occurred at concentrations of 0.5 μg/mL. For *Streptococcus pneumoniae*, a major cause of upper respiratory tract infections, teixobactin acted at concentrations lower than 0.03 μg/mL. This new molecule, besides being a great help for the current therapeutic arsenal, clearly shows that the new methodology opens up a wide front for the discovery of new antibiotic molecules of natural origin.

8. Conclusions

It is a whole new world where the humanity thrives today. With the astonishing increase of bacterial resistance to conventional chemical antibiotics, added to the possibility of these losing their effectiveness during the next 5 years, it is absolutely essential

to develop alternative antimicrobial strategies, in order to fight this serious problem faced by society today.

Very recently, in February 2016, Liu et al. (2016) published an article showing a mechanism of resistance to colistin mediated by plasmids (mcr-1), allowing the horizontal transference of resistance genes, and greatly facilitating its dissemination. This discovery was made in China, in a sample of *Escherichia coli* collected from pork for human consumption. The discovery arouses great concern since, beyond degrading the last antibiotic still with action in Gram-negative bacteria, its dissemination should be very fast because of its plasmid origin, allowing for the horizontal transference of these genes. It should be remembered at this point that China is the largest producer of pigs for human consumption, with a production of 57 million tons in 2014, and also is one of the largest users of colistin for agricultural purposes (Liu et al., 2016). All this only reinforces the need to control more and more the use of antibiotics and to perform research for new alternatives for the treatment of infectious diseases.

Currently, there are already several alternative strategies, such as antibiotherapy with phages and lysins, and both show a high bacterial specificity, a suitable spectrum of action and, most importantly, do not affect the commensal flora of the host. Additionally, there are countless antimicrobial compounds being tested in clinical trials, while other compounds are still under development. However, and although such antimicrobial alternatives exhibit several advantages over conventional chemical antibiotics, it is imperative to know more about the pharmacokinetics and pharmacodynamics of these therapeutic strategies *in vivo*.

Until now, only a few AMPs have received approval by both FDA and EMEA, and their uses are restricted to topical applications due to both their short half-life and the high concentrations in which they are administered.

In spite of bacteriocins being an excellent therapeutic alternative to chemical antibiotics, the pharmaceutical industry remains reluctant to finance research and production of bacteriocin preparations, mainly due to the high production costs involved and the instability of the final (formulated) products. Bacteriocins produced by probiotic bacteria are already available on the market, but they are considered more a therapeutic complement than a therapeutic alternative.

Vaccination is one of the most promising strategies both at a preventive level, as well as in terms of cost-effectiveness, but do not allow to fight established bacterial infections and it is still limited to certain strains of pathogenic bacteria. Furthermore, in a near future, with genomics and bioinformatics providing innovative methods to the development of new antimicrobial vaccines specific to certain pathogenic bacteria, these alternatives can reduce both the rate of bacterial infections at the community and the need for chemical antibiotics.

Nowadays, through genetic engineering and recombinant DNA technology, it is possible to develop transgenic phages, lysins and bacteriocins, in order to increase their ability to infect biofilms, make them more specific and stable, with a wider spectrum of action and increasing both their potency and efficacy. To conclude, deepening the current knowledge about these innovative therapeutic strategies is of utmost importance. While such innovative therapeutic strategies are not defined, structured and available in the market, it is imperative to develop standards in order to control the use of current chemical antibiotics. In addition, it is indispensable that health professionals use, as a last resort, new chemical antibiotics that may arise in the market in the near future, preventing in this way the spread of bacterial resistance to them.

Transparency declarations

None to declare.

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