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Preface

Microbial infections still represent one of the major causes of mortality and morbidity worldwide. Irrational usage of antimicrobials has lead to increased resistance, causing clinical, social and economical disabilities. Therefore, one of the major challenges of scientists is to develop novel alternative methods to handle infections and reduce resistance and other side effects produced by the actual therapies. The aim of this book is to offer a perspective on novel approaches of infection control by using naturally-derived products in order to modulate the virulence of pathogens, without the risk of developing resistance. We intend to highlight the utility of microbial-, vegetal- and animal-derived compounds with potential antimicrobial activity by exploiting their effect on microbial virulence. Furthermore, this book aims to reveal the potential to assimilate recent bio-technological findings, like the usage of nanotechnology as efficient shuttles for stabilizing, improved targeting and the controlled release of natural products in order to efficiently fight infections.

1 Introduction

Severe infections caused by antimicrobial resistant microorganisms are present in all parts of the world and still represent a main cause of disability and death. *Antimicrobial resistance* (AMR) is defined as the resistance of a microorganism to an antimicrobial drug that was originally effective for the treatment of infections caused by that microorganism. AMR is a broad term and it should not be confounded with *antibiotic resistance,* which refers specifically to the resistance to antibiotics that occurs in common bacteria that cause infections. AMR encompass resistance to drugs used to treat infections caused also by other types of microorganisms, such as parasites, viruses and fungi.

Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness, higher health care expenditures, and a greater risk of death. Usually, the death rate for patients with serious infections caused by resistant bacteria is about twice that of patients with infections with the non-resistant variant of the same bacteria (WHO, 2014a). Although we are aware of this increasingly serious threat to global public health, the efforts made by government sectors and society is yet unable to limit its rapid spread. New resistance mechanisms constantly emerge and spread globally threatening our ability to treat common infectious diseases, which leads to the failure of many standard medical treatments. Many pathogens have now become resistant to more than one antimicrobial, being generically called multidrug resistant pathogens (MDR) or *superbugs*. For example, in the last year, more than 450 000 new cases of multidrug-resistant tuberculosis (MDR-TB) were identified, while extensively drug-resistant tuberculosis (XDR-TB) has been identified in 92 countries (WHO, 2014b). XDR-TB is a form of tuberculosis caused by bacteria that are resistant to some of the most effective anti-TB drugs and has developed after the mismanagement of individuals with multidrug-resistant TB (MDR-TB). Individuals infected with microbial MDR strains have an increased death rate. For example, studies reported that individuals with MRSA (meticillin resistant *Staphylococcus aureus*), *Pseudomonas aeruginosa*, *Klebsiella* sp., *Clostridium difficile* and *Acinetobacter* sp., which are common sources of severe infections in hospitals, are estimated to be 64% more likely to die than patients with a non-resistant form of the infection (Pastagia *et al.*, 2012). Although patients with chronic diseases, burns and immunosuppressive conditions are most susceptible to acquiring difficult to treat infections, most people are prone to infections with MDR microorganisms. One explanation of this high risk of infection with resistant pathogens relies on the fact that in the last decades the use of antimicrobials was highly irrational. Therefore, the activity of medical units, industrial complexes, pharmaceutics and animal breeding facilities lead to the release and accumulation of high amounts of antimicrobial compounds in the environment. These pollutants have exerted a selective pressure on microorganisms, facilitating the selection of the highly resistant strains. Microorganisms rapidly spread the acquired resistance genes *via horizontal gene transfer*, and lead to severe

community and healthcare acquired infections (McBain *et al.*, 2002; Tammy, 2014). Infections that are detected within the first 48 h of hospitalization are defined as *community-acquired infections* (CAIs), whereas infections that occur later during the course of hospitalization are defined as *nosocomial*, or *hospital-acquired infections* (HAIs). Conceptually, the difference between the 2 types of acquisition is based on the ecologic environment and the presence or absence of medical interventions or devices at the time of acquisition of the bacteremia (Siegman-Igra *et al.*, 2002).

Recent statistics revealed that almost 70% of global healthcare-associated infections are caused by resistant pathogens, and the percentages are slightly different depending on the continent and country. Highest prevalence rates of resistance in major bacterial pathogens was seen in Asia. For example, high-level macrolide resistance in *Streptococcus pneumoniae* was reported to be higher than 70% among clinical isolates from Asian countries such as Korea, China, Japan, Hong Kong, Thailand and Vietnam. The prevalence rates of multidrug-resistance in *Acinetobacter* spp. were higher than 80% in Thailand, Malaysia and India. Some European countries, such as Austria, have a well consolidated situation as compared with other areas, but still many efforts are necessary to implement an effective mechanisms to prevent and control antimicrobial resistance worldwide (ABR, 2013).

In healthcare facilities, most infections are due to resistant pathogens such as MRSA and multidrug-resistant Gram-negative bacilli. The most severe healthcare-associated infections are device – and procedure – associated; therefore, recent antimicrobial approaches try to control and limit the spread of those particular infections (Beardsley *et al.*, 2006; Hashemi *et al.*, 2012; Williams, 2001).

As opposed to HAIs, CAIs are acquired infectiously from normal social contact in the community. Although most infection control measures are focused on hospitals, recent studies reveal the urgent need for more targeted interventions to prevent the spread of drug-resistant microorganisms in nursing homes, as community-associated strains of methicillin-resistant *Staphylococcus aureus* (CA-MRSA) are on the rise at these facilities (Murphy *et al.*, 2013). Furthermore, it has been demonstrated that many HAIs and CAIs etiologies are related genetically. An important cause for the spread of antimicrobial resistance is the failure to apply efficient infection control measures in healthcare settings and outside (Giedraitiene *et al.*, 2011). Because of these findings and despite the clear and accepted classification of infections depending on the origin, recent studies reveal that many times inconsistent criteria are used to define community-acquired, healthcare-associated and hospital-acquired infections (Henderson *et al.*, 2013). Therefore, as a first step for an efficient infection control, all infections should be carefully analyzed and classified, through rigorous and standardized approaches. For resistant etiologies, the exact resistance mechanisms should be established for choosing the best therapeutic approach.

The purpose of this book is to reveal a perspective on novel approaches in handling infections by using naturally-derived products in order to modulate the virulence of pathogens while avoiding the risk of resistance development. We intend to highlight

the utility of microbial, vegetal and animal – derived compounds with potential antimicrobial activity by exploiting their effect on microbial virulence. Furthermore, this book aims to reveal the potential to assimilate recent bio-technological findings, such as the implication of nanotechnology to develop efficient shuttles for stabilizing, targeting and improving the controlled release of natural products in order to efficiently fight infections.

1.1 Resistance Mechanisms and Spreading

Bacterial resistance is closely associated with the use of antimicrobial agents in clinical practice and industrial processes. Prolonged therapy with antibiotics leads to the development of several mechanisms that confer microorganisms with resistance to a particular type or class of antibiotics and/or other antimicrobial agents. There are two main types of resistance: *innate* and *acquired* (adapted).

Innate resistance or naturally occurring antibiotic resistance is a common phenotype. The genes that confer this type of resistance are known as the environmental *resistome*, and may be transferred from non-disease-causing bacteria to pathogens, leading to clinically significant antibiotic resistance (Wright, 2010). Studies have revealed that antibiotic resistance genes existed within the microbial genome prior to the discovery of antibiotics and that they may have evolved by random mutations or by gene transfer between microorganisms that normally produce antibiotics and different pathogens (Nelson, 2009). Nevertheless, there is evidence that heavy metals and some pollutants may select for antibiotic-resistant bacteria, generating a constant source of naturally occurring resistant microorganisms in small amounts (Toner, 2005).

Acquired resistance may be obtained by two different ways:

- Mutations in chromosomal genes leading to vertical gene transfer and cross-resistance,
- Gene transfer from one microorganism to another through: i) plasmids (conjugation or transformation), ii) transposons (conjugation), iii) integrons and iv) bacteriophages (transduction) (Giedraitiene *et al.*, 2011).

Most resistance mechanisms are encoded by plasmids, which are potentially transmissible to other bacteria. Regardless of the mechanism of resistance acquisition, the emergence of a phenotype resistant to antimicrobials depends on various factors of the microbial host such as the degree of resistance expression, capability of a microorganism to tolerate a certain resistance mechanism, initial colonization site, and others.

In antibiotic resistant bacteria, there are several mechanisms of resistance. These mechanisms can i) modify the chemical structure of the antibiotic, ii) avoid its accumulation through physical removal from the cell, iii) modify the target site so that it is not recognized by the antibiotic anymore, or iv) overproduce the target leading to the

titration of the antibiotic (Toda, 2014). The most important resistance mechanisms are presented in Figure 1.1.

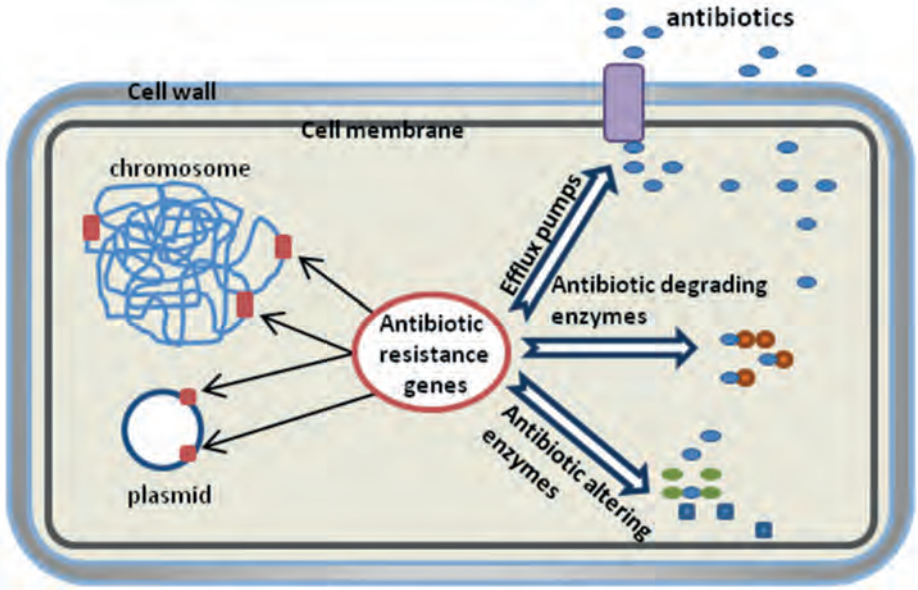

Figure 1.1: Illustration of the main antibiotic resistance mechanisms: overproduction of efflux pumps, in order to remove the antibiotic from the bacterial cell; the production of antibiotic degrading enzymes to inactivate the antibiotics; and the production of altering enzymes that modify the structure of antibiotics.

Depending on their cellular target, antibiotics may be clustered within the following groups:

- Antibiotics that interfere with cell wall synthesis: penicillins, cephalosporins, vancomycin, ß-lactamase inhibitors, carbapenems, aztreonam, polymycin, bacitracin;
- Protein synthesis inhibitors:
- inhibiting 30s ribosomal subunit: aminoglycosides (gentamicin), tetracyclines;
- inhibiting 50s ribosomal subunit: macrolides, chloramphenicol, clindamycin, linezolid, streptogramins;
- DNA synthesis inhibitors: fluoroquinolones, metronidazole;
- RNA synthesis inhibitors: rifampin;
- Mycolic acid synthesis inhibitors: isoniazid;
- Folic acid synthesis inhibitors: sulfonamides, trimethoprim (Li & Nikaido, 2009).

Because of the variability of available antibiotics, microorganisms have developed different resistance mechanisms. The main types of resistance mechanisms are summarized in the Table 1.1, along with the class of antibiotics and the cellular target.

In recent years, the introduction of antibiotics into the environment through human waste (medication, farming), animals, and the pharmaceutical industry has been a source of concern. Resistant bacteria follow the antibiotic waste discharge, thus introducing antibiotic-resistant bacteria into the environment that are able to replicate fast and spread the resistance genes to other bacteria. Therefore, even if the specific antibiotic is no longer introduced into the environment, antibiotic-resistance genes will persist through the bacteria that have since replicated without continuous exposure (Martinez, 2009).

Table 1.1: The cellular target and the acquired resistance mechanisms for the main classes of antibiotics.

Antibiotics	Cellular target	Resistance mechanism
β-lactams Vancomycin	Cell wall	Reduced binding affinity
Daptomycin	Cell membrane	Altered interaction with cell membrane
Fluoroquinolones Rifamycins	DNA/RNA synthesis	Target modification
Trimethoprim Sulfonamides	Folate synthesis	Overproduction of antibiotic target
Linezolid Tetracyclines Macrolides Aminoglycosides	Protein synthesis	Target modification

1.2 Resistant Pathogens

The most investigated resistant microorganisms are those with the greatest clinical impact.

1.2.1 *Staphylococcus aureus*

S. aureus, found on the mucous membranes and the human skin of around a third of the population is one of the major resistant pathogens. This gram-positive bacterium is extremely adaptable to antibiotic pressure and is one of the earliest bacteria found to be resistant to penicillin in 1947, just four years after the drug started being mass-produced. In order to overcome this issue methicillin and then oxacillin were introduced into clinical practice. First detected in 1961, methicillin-resistant *S. aureus* (MRSA) is now one of the most common etiologies in hospitals. It has been estimated that half of all *S. aureus* infections are resistant to penicillin, methicillin, tetracycline and erythromycin, which left vancomycin as the only effective agent available at the time (Bozdogan *et al.*, 2003). Meanwhile several strains with intermediate levels of resistance, termed glycopeptide-intermediate *S. aureus* (GISA) or vancomycin-intermediate *S. aureus* (VISA), have been reported since late 1990s. First documented strain with complete (> 16 μg/mL) resistance to vancomycin, termed vancomycin-resistant *S. aureus* (VRSA) was reported in the United States in 2002. Although a new class of antibiotics, oxazolidinones, became available in the 1990s, and the first commercially available oxazolidinone, linezolid, is comparable to vancomycin in effectiveness against MRSA, linezolid-resistance in *S. aureus* was reported in 2001 (Tsiodras *et al.*, 2001). These statistics and the slow progress in novel antibiotic development demonstrate the acute need for finding alternative anti-pathogenic approaches.

1.2.2 *Streptococcus* sp.

The group A Streptococcus, *Streptococcus pyogenes*, produces infections that can be usually treated with current antibiotics, depending on the severity of infection. Although there are no reports published suggesting the existence of penicillin resistance in *S. pyogenes*, the increasing rates of macrolide-resistant group A streptococci (MRGAS) pose considerable clinical problems in many countries. A recent ecologic study revealed that Streptococcal resistance is directly associated with antibiotic selection pressure on a national level (Albrich *et al.*, 2004).

Streptococcus pneumoniae is responsible for a wide range of infections such as pneumonia, bacteremia, otitis, meningitis, sinusitis, peritonitis and arthritis. Increased resistance to penicillin and other ß-lactams has been documented with *S. pneumoniae* worldwide. The major mechanism of resistance involves the introduction of mutations in genes encoding penicillin-binding proteins. Penicillin-resistant *S. pneumoniae* and macrolide-resistant *S. pneumoniae* are markers of resistance to antibiotics commonly used as first-line drugs for respiratory tract infections (Albrich *et al.*, 2004).

1.2.3 *Enterococcus* sp.

Enterococci are well equipped with a variety of intrinsic, naturally occurring antibiotic resistances, but they are also capable of acquiring new resistance genes and/or mutations. Many nosocomial infections are associated with multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium*. The most frequent resistance phenotypes in this genus are: penicillin-resistant Enterococcus, first observed in 1983, vancomycin-resistant *Enterococcus*, isolated in 1987, and linezolid-resistant *Enterococcus* documented in the late 1990s (Hidron *et al.*, 2008). The combination of high-level resistance to ampicillin, vancomycin, and aminoglycosides is now common among hospital-acquired *Enterococcus faecium* infections.

1.2.4 *Clostridium difficile*

Clostridium difficile is a nosocomial pathogen that causes diarrheal disease in hospitals worldwide. This species is incriminated for at least 14,000 deaths each year in the American continent only (Frieden, 2013). The most important risk factor for the outbreaks of bacterial infections produced by *C. difficile* is represented by the overuse of antibiotics in the raising of livestock. *C. difficile* colitis is usually associated with fluoroquinolones, cephalosporins, carbapenems, and clindamycin use. For this reason, it is recommended that fluoroquinolones and clindamycin to be avoided in clinical practice due to their association with *C. difficile* infections (Baxter *et al.*, 2008). Clindamycin-resistant *C. difficile* was reported as the causative agent of large outbreaks of diarrheal disease in many hospitals since 1989. Geographically dispersed outbreaks of *C. difficile* resistant to fluoroquinolones, such as ciprofloxacin and levofloxacin, were also reported in 2005, especially in North America (Johnson *et al.*, 1999; Loo *et al.*, 2005).

1.2.5 *Pseudomonas aeruginosa*

The wide spread opportunistic pathogen *Pseudomonas aeruginosa* is characterized by a very low antibiotic susceptibility, which is attributable to a concerted action of multidrug efflux pumps with chromosomally encoded antibiotic resistance genes (for example, *mexAB-oprM*, *mexXY*, etc.) and the low permeability of the bacterial cellular envelopes (Poole, 2004). Although the import of resistance mechanisms using mobile genetic elements is usually the greatest concern, the most difficult challenge clinicians face with *P. aeruginosa* is its ability to rapidly develop resistance during the course of treating an infection. The chromosomally encoded AmpC cephalosporinase, the outer membrane porin OprD, and the multidrug efflux pumps are particularly relevant markers for the current therapeutic approaches (Lister *et al.*, 2009). The most

extensively *P. aeruginosa* resistant strains were isolated from cystic fibrosis patients and are able to produce microcolonies or biofilms, embedded within a polysaccharide matrix, which makes them tolerant to high amounts of antimicrobials (Lambert, 2002). Furthermore, the ability to produce and regulate the production of alkyl quinolone Quorum Sensing signaling molecules has recently proved to impact on antibiotic resistance in this versatile pathogen (Nguyen *et al.*, 2011).

1.2.6 *Acinetobacter* spp.

Multidrug-resistant *Acinetobacter baumannii* is one of the most difficult antimicrobial-resistant gram-negative bacilli to control and treat. This species produces outbreaks of infection and health care – associated infections, including bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection (Maragakis & Perl, 2008). *Acinetobacter* spp. has developed specific mechanisms of resistance to all existing antibiotic classes and has the capacity to acquire new determinants of resistance. Resistance to ß-lactams is related with the production of 4 classes of ß-lactamases, changes to the outer membrane proteins and overproduction of efflux pumps, similar to *P. aeruginosa*. In addition to efflux pumps, the resistance to aminoglycosides is mediated mainly by aminoglycoside-modifying enzymes, while resistance to quinolones is often caused by modifications in the structure of DNA gyrase, secondary to mutations in the quinolone resistance-determining regions of the *gyrA* and *parC* genes. Resistance to tetracyclines is characterized by two different mechanisms: i) transposon-mediated efflux pumps and ii) ribosomal protection protein, which shields the ribosome from the action of tetracycline. Resistance to polymyxins is related with modifications in the lipopolysaccharide of *A. baumannii* and nowadays resistance to colistin is wide spread in hospitals (Perez *et al.*, 2007).

1.2.7 *Escherichia coli*

Normal intestinal microbiota is a reservoir for resistance genes; the prevalence of resistance in commensal *Escherichia coli* is a useful indicator of antibiotic resistance in bacteria. Antibiotic resistance in *E. coli* strains carried and acquired in the community is rapidly rising; especially resistance to critically important antibiotics, broad-spectrum resistance strains being reported both in community and hospitals (Colligno, 2009). The genetic mechanisms that lead to bacterial resistance are multiple and their spread in different bacterial populations is enabled by several efficient transfer systems of mobile genetic elements. During recent years, the importance of integrons (mobile gene expression systems) for the dissemination of resistance in *E. coli* is one of the most investigated mechanisms. Because of its epidemiological importance, the

prevalence and nature of antimicrobial resistance in zoonotic Shiga toxin-producing *E. coli* is of a great interest (Guerra *et al.*, 2006).

A recent study reported that a very resistant *E. coli* strain known as sequence type ST131 is wide spread in hospitals and long-term care facilities in the US. This strain is commonly associated with fluoroquinolone resistance and its expansion is recognized as pandemic (Banerjee, 2013).

1.2.8 *Salmonella* spp.

Strains of *Salmonella* spp. with enhanced resistance to antimicrobial drugs are now widespread in both developed and developing countries. Recent studies demonstrate that many strains are zoonotic and acquire their resistance from the food-animal host before onward transmission to humans through the food chain (Threlfall, 2002). The number of antimicrobial-resistant Salmonella serotypes is slowly increasing in recent years, and drug-resistant Salmonella continues to pose a public health threat worldwide, particularly as resistance spreads across multiple classes of drugs. This requires the use of more expensive drugs, makes treatment less effective, and, in the worst case scenario, leaves infections untreatable. Cephalosporin resistance is the biggest current issue in drug-resistant Salmonella. At present, about five percent of the strains resistant to cephalosporins belong to *Salmonella* Heidelberg and *Salmonella* Newport strains (Krietsch, 2013). Multidrug-resistant (MDR) *Salmonella enterica* (serotypes typhi and paratyphi A) has become an emerging problem in endemic countries and the resistance to oral antibiotics including ampicillin, chloramphenicol, cotrimoxazole (trimethoprim-sulfamethoxazole), ofloxacin, and ciprofloxacin is increasing across all endemic areas.

1.2.9 *Klebsiella pneumoniae*

Klebsiella spp. counts amongst most common pathogens isolated in hospitals, especially in intensive care units and *K. pneumoniae* is the most frequently encountered carbapenemase-producing Enterobacteriaceae. *K. pneumoniae* exhibits numerous mechanisms of antibiotic resistance, many of which are located on highly mobile genetic elements. Carbapenem antibiotics (often the treatment of last choice for resistant infections) are generally not effective against *K. pneumoniae* carbapenemase -producing strains (Sanchez *et al.*, 2013). Due to the limited efficiency and high toxicity of the alternative agents, many clinical experts recommend using a combination therapy instead of monotherapy in patients infected with *K. pneumoniae* carbapenemase -producing strains (Petrosillo *et al.*, 2013).

1.2.10 *Mycobacterium tuberculosis*

Tuberculosis (TB) is a severe disease, increasing across the globe, especially in developing countries. Tuberculosis resistant to antibiotics is called MDR TB (Multidrug Resistant TB) and it causes more than 150 000 deaths annually across the world. Studies have revealed that severe chronic diseases and those that impair the normal immunity (as the rise of the HIV/AIDS epidemic) have contributed to this huge number of mortal cases (Dalton *et al.*, 2012). TB was considered one of the most prevalent diseases, and did not have a cure until the discovery of Streptomycin by Selman Waksman in 1943. However, the bacteria soon developed resistance and since then drugs such as isoniazid and rifampin have been used to treat tuberculosis. Resistance of *M. tuberculosis* to isoniazid, rifampin, and other common treatments has become an increasingly relevant clinical challenge. The main mechanism of drug resistance in *M. tuberculosis* seems to occur by spontaneous mutations in its genome (Gao & Li, 2010; LoBue, 2009).

1.2.11 *Neisseria gonorrhoeae*

N. gonorrhoeae is a sexually transmitted pathogen that can cause pelvic pain, pain on urination, penile, and vaginal discharge, as well as systemic infections. Although the treatment with penicillin has proved to be helpful at the beginning, since late 1970s resistant strains have begun to emerge. Penicillin resistance mechanisms in this species has developed through two mechanisms: i) chromosomally mediated resistance (involves stepwise mutation of *penA* gene, which codes for a penicillin-binding protein; *mtr* gene, which encodes an efflux pump to remove penicillin from the cell; and *penB*, which encodes bacterial cell wall porins), and ii) penicillinase-mediated resistance (which involves the acquisition of a plasmid-borne ß-lactamase) (Deguchi *et al.*, 2010). Next, fluoroquinolones were used as the next-line treatment, but resistance to these drugs was achieved through efflux pumps and mutations to the *gyrA* gene, which encodes a DNA gyrase. Third-generation cephalosporins have been used to treat gonorrhoea since 2007, although resistant strains have since emerged. Strains of this species have also been found to be resistant to tetracyclines and aminoglycosides and this is explained by the fact that *N. gonorrheoea* has a high affinity for horizontal gene transfer. Today, the most used treatment for infections caused by *N. gonorrheoea* is represented by injectable ceftriaxone, sometimes in combination with azithromycin or doxycycline (del Rio *et al.*, 2012).

1.3 Current Antimicrobial Therapies

The development of antimicrobial resistance among pathogens has been progressive and relentless. Gram-negative pathogens of particular concern include

extended-spectrum β-lactamase – producing Enterobacteriaceae, carbapenem-resistant Enterobacteriaceae and multidrug-resistant *P. aeruginosa* (Kanj & Kanafani, 2011), while the most investigated gram-positive resistant microorganisms are methicillin-resistant *S. aureus*, penicillin-resistant Pneumococci, and vancomycin-resistant Enterococci (Rivera & Boucher, 2011). Treatment of diseases caused by resistant bacteria requires appropriate use of available antibiotics and stewardship to prolong their effectiveness. In addition, appropriate and aggressive infection control efforts are vital to help prevent the spread of resistant pathogens.

1.3.1 Current Concepts in Antimicrobial Therapy against Resistant Gram-positive Bacteria

Because of the great variability of resistance mechanisms, specific therapeutic approaches should be considered for particular infections. Despite increasing knowledge about resistance transmission patterns and new antibiotics, these organisms continue to cause significant morbidity and mortality, especially in the health care settings. However, a community-associated resistant infection, such as methicillin-resistant *S. aureus* (CA-MRSA) has also been described in patients with no previous contact with the health care environment. Unlike hospital-associated MRSA, many CA-MRSA strains are susceptible to gentamicin, tetracyclines, lincosamides, and trimethoprim-sulfamethoxazole. New challenges in treating infections produced by organisms that are more resistant include *S. aureus* with heteroresistant vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *S. aureus*, and MRSA resistant to linezolid and daptomycin (Rivera & Boucher, 2011). The treatment of uncomplicated CA-MRSA infections in immunocompetent hosts consists of making an incision and drainage, local debridement, or abscess drainage alone. On the other hand, for patients with signs of systemic illness or comorbidities, empirical treatment which includes antibacterial therapy should be given. Successful clinical outcomes have been obtained when oral antibiotics, including trimethoprim-sulfamethoxazole, doxycycline, and clindamycin were given. Strains resistant to erythromycin and susceptible to clindamycin should always be tested for inducible clindamycin resistance (*via* the D-test) as many treatment failures have been reported. Linezolid is not recommended to treat uncomplicated infections caused by *S. aureus* because of the associated toxicity and costs (Rivera & Boucher, 2011). Treatment of MRSA infections in patients with systemic diseases or immune disorders includes monotherapy with intravenous antibiotics in addition to prompt and thorough incision and drainage of abscesses, as well as debridement of wounds. Vancomycin may be used at a dosage of 10 to 15 mg/kg intravenously every 12 hours adjusted for renal function. Other clinical options include linezolid, 600 mg administered intravenously every 12 hours, with the limitations related to high costs and toxicity. Daptomycin is also effective for the therapy of MRSA infections at a dosage of 4 mg/kg daily. New antimicrobial agents include telavancin, approved by the

US Food and Drug Administration in 2009 at the dosage of 10 mg/kg daily for patients with normal renal function, and ceftaroline, approved by FDA in 2010 for the treatment of acute *S. aureus* at a dosage of 600 mg intravenously every 12 hours in patients with normal renal function. However, additional caution should be taken for the new drugs since they have a high toxicity and also the increased cost limits their use (Rybak *et al.*, 2009; Wilcox *et al.*, 2010).

In *S. pneumoniae* infections, the treatment of non-central nervous system infection caused by resistant pneumococci still relies on penicillins, aminopenicillins, and third-generation cephalosporins. On the other hand, for the treatment of meningitis a combination of vancomycin and a third-generation cephalosporin is recommended, due to concerns about the emergence of penicillin or cefotaxime non-susceptible pneumococcal isolates (Cunha, 2006). Macrolide monotherapy is not recommended as empirical treatment of community acquired pneumonia, especially in geographic areas where the prevalence of resistant *S. pneumoniae* strains is high. Starting in 2001, isolates resistant to fluoroquinolones were obtained and this phenotype seems to be related with the rapid increase in resistance associated with clonal dissemination and the wide use of quinolones worldwide (Fuller & Low, 2005).

Antibiotic resistance among Enterococci is often conferred through mutation and acquisition of genetic material from other species. Enterococci have acquired resistance to penicillin and vancomycin and one of the main challenges for physicians treating vancomycin resistant enterococci (VRE) is their intrinsic resistance to many antibiotics, including β-lactams, aminoglycosides, lincosamides, and trimethoprim-sulfamethoxazole (Gold, 2001). Results revealing the efficacy of novel antibacterial agents used in the management of VRE infections are very limited and most of these drugs are not approved by the FDA. Tetracycline, doxycycline, oral novobiocin with ciprofloxacin, and doxycycline have been reported as effective in treating VRE infections, even though there are no clinical studies to support these therapies (Linden & Miller, 1999).

1.3.2 Current Concepts in Antimicrobial Therapy against Resistant Gram-negative Bacteria

The medical literature contains many studies illustrating the global increase in the burden of antimicrobial resistance among gram-negative pathogens, but wide regional differences exist, accentuating the need to take into account local epidemiology when making decisions about empirical therapy for serious infections (Pfeifer *et al.*, 2010).

Recently, a consensus was proposed to describe different resistance phenotypes of Gram-negative bacteria (GNB):

– *Multidrug-resistant* (MDR), which describes an isolate that is non-susceptible to at least one agent in at least three antimicrobial categories, that are potentially active against the respective GNB;

- *Extensively drug-resistant* (XDR), which describes isolates non-susceptible to at least one agent in all but two or fewer antimicrobial categories, that are potentially active against the respective GNB;
- *Pandrug-resistant* (PDR) bacteria, defined as non-susceptibility to all agents in all antimicrobial categories for that isolate (Zavascki *et al.*, 2013).

Production of β-lactamase is the most commonly encountered mechanism of resistance of bacterial pathogens to β-lactam antibiotics. Carbapenemases are the β-lactamases with the widest spectrum of activity, because they are active against most other members of the β-lactam family with few exceptions. Carbapenem resistance has become a major global health concern since 2000 when the emergence of metallo-β-lactamases VIM, IMP and NDM (molecular class B), OXA-48 and its derivatives (molecular class D), and *Klebsiella pneumoniae* carbapenemases (KPCs, molecular class A) have rapidly caused several paradigm shifts in antibiotic therapy against gram negative bacteria (Livermore & Woodford, 2000).

Combination therapy for GNB is usually based on a cornerstone antibiotic for which the organism presents *in vitro* susceptibility, although this is likely not possible for PDR isolates. Carbapenems are considered first-line agents in treating infections caused by Extended-Spectrum β-Lactamase – Producing Enterobacteriaceae (ESBL). Current therapy consists of administration of imipenem at 500 mg intravenously (IV) every 6 hours up to 1 g IV every 8 hours in serious infections or meropenem at 1 g IV every 8 hours. Recent data have shown that ertapenem and doripenem, the newest addition to the carbapenem class, exhibit an activity against ESBL-producing pathogens that is similar to that of imipenem and meropenem (Lye *et al.*, 2008).

Currently, polymyxins represent the antibiotic class for which most carbapenemase producing GNB reveled *in vitro* susceptibility, and polymyxin-only-susceptible isolates account for a significant proportion of GNB with XDR profile (Zavascki *et al.*, 2013). Colistin and polymyxin B are the most common cornerstone agents in combination schemes and lately other agents such as tigecycline have also become the preferred antibiotics in some combination schemes for *A. baumannii* and Enterobacteriaceae infections. The most frequently used adjuvant therapies for carbapenemase producing GNB infections are carbapenems, tigecycline, fosfomycin, aminoglycosides and rifampicin (Zavascki *et al.*, 2013).

1.4 Limitations of Current Antimicrobial Therapies

A primary concern when using antibiotics is the development of bacterial resistance that may be influenced not only by the widespread use of antibiotics, but also by antibacterial pharmacokinetics, such as maintenance of insufficient bactericidal concentrations that reach the site of infection. In addition, poor adherence to prescribed

regimens that require frequent administration, along with undesirable adverse events, affect the development of bacterial resistance and the success of treatment regimens.

A major limitation of current antimicrobial approach is that there are a limited repertoire of drugs active against fungi (particularly *Aspergillus* sp.) as well as certain viruses (as for example, cytomegalovirus) and there may be observed a low ability to eradicate certain sites of infection (for example, Pseudomonas related pneumonia, different MRSA infections) even with effective agents (Pizzo & Young, 1984). The high antibiotic resistance rate observed nowadays is related with the irrational use of antibiotics by an increasing population and with the ability of bacteria to adapt in an overwhelming manner. Furthermore, the fact that some countries still allow the use of antibiotics in animals food, being covered by the fact that the increasing population of Earth needs more food, strongly contribute to the alarming increase in antibiotic resistance rates.

All antimicrobial drugs have the potential to harm the host, because of their high toxicity and the reduction of normal microbiota. The most severe symptoms include, nervous system symptoms (pain, tingling and numbness, dizziness, malaise, weakness, headaches, anxiety and panic, loss of memory, psychosis), musculoskeletal symptoms (tendon ruptures, tendonitis, weakness, joint swelling), sensory symptoms (tinnitus, altered visual, olfactory, and auditory function), cardiovascular symptoms (tachycardia, shortness of breath, chest pain, palpitations), skin reactions (rashes, hair loss, sweating, intolerance to heat or cold) and gastrointestinal symptoms (nausea, vomiting, diarrhea, abdominal pain) (Liu *et al.*, 2011) etc.

Current therapies are ineffective mainly on invasive infections, particularly those involving persistent bacteraemia, necrotizing pneumonia, osteomyelitis and other deep-seated sites of infections, which are associated with high mortality. The ideal antibiotic for these infections does not yet exist, but such an agent should have the following properties: rapid bactericidal effect; excellent tissue penetration; consistent pharmacokinetics and pharmacodynamics that allow for predictable dosing; low potential for the development of resistance while on therapy; low side effect profile; and demonstrated clinical and microbiological efficacy (Nguyen & Graber, 2010). Although several combinations of antibiotic therapies have increased the bacterial clearance, preventing the emergence of resistance and ensuring delivery of adequate drug to sites of infection; recent studies suggest that the toxin release and subsequent host inflammation in infections caused by microbial clones that overproduce toxins as a major virulence factor should additionally benefit from therapies which may attenuate toxin production (Stevens *et al.*, 2007). As research advance deeper into the mechanisms of the infectious process, it is revealed that virulence represents one of the main factors, which should be considered for future therapies.

2 Microbial Virulence

Pathogenic bacteria manifest two characteristic traits: pathogenicity and virulence. *Pathogenicity* is the ability of a pathogen to generate an infectious process upon entering a susceptible host, which is clinically constant. While pathogenicity is a species characteristic, virulence is strain related. *Virulence* is the relative property of a pathogenic strain to colonize, multiply and eventually invade host cells and tissues and produce toxins, determining a pathologic condition of the host (Chifiriuc & Mihaiescu, 2011). The virulence quantitatively measures the level of pathogenicity of a bacterial strain for a certain host and depends on several structural and physiological particularities of the pathogen. Virulence is a multifactorial trait and it relies on the following traits of pathogenic bacteria: i) infection potential (the ability of a bacterial strain to colonize a host), ii) aggressive potential (also referred to as invasion of the host cells and tissues), and iii) toxigenic potential (the ability of the strain to produce and release toxins).

2.1 Virulence Factors

Virulence is correlated with the presence of several cell-attached and soluble bacterial structures, which are called *virulence factors* or *virulence determinants*. Virulence factors refer to the properties (i.e., gene products) that enable microorganisms to establish on or within a host of a particular species and enhance their potential to cause disease. Virulence factors include bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium and and its hydrolytic enzymes that may contribute to the pathogenicity of the bacterium. The molecules that modulate virulence are classified into 4 groups:

- Adhesion molecules (proteins which mediate the interaction of bacterial cells with host cells),
- Invasion related molecules (bacterial proteins which allow them to enter host cell),
- Molecules responsible for the aggressiveness of the bacterial strain (molecules which produce tissue lesions and favors the spread of infection such as toxins or proteases),
- Molecular modulators (bacterial molecules that inhibit the host defense mechanisms or stimulate the production of certain host molecules involved in immunity with incompletely elucidated consequences).

The interaction between bacterial virulence factors and host immune effectors dictates the evolution of the infectious process. Depending on the environmental conditions some pathogens, especially *opportunistic pathogens* (which may be commensals which are manifesting their pathogenic potential in certain conditions) may be “quiet”

colonizers, usually in chronic infections, or very virulent pathogens, usually during acute infections.

2.1.1 Cell-attached Virulence Determinants – Bacterial Adhesins

Adherence is mediated by specific structures of the bacteria cell surface, known as adhesins. These structures are mainly adaptive and they may be lost from the bacteria surface after several passages *in vitro*. Chemically, adhesins may be proteins and non-proteins. The typical bacterial adhesions are fimbria, pili, flagella and capsule.

2.1.1.1 Fimbriae and Pili

Fimbriated and piliated bacteria are able to adhere to the host epithelial cells and to agglutinate erythrocytes in a manner resembling classical hemagglutination. A *pilus* is a hair-like protein structure, typically 7 to 8 nm in diameter, on the surface of the cell, especially in the case of gram-negative bacteria. Several hundred pili can extend from the surface of a bacterial cell.

A *fimbria* is defined as a short pilus. They are used for the attachment of bacterial cells to a surface or other cells, and are either located at the poles of a cell, or are evenly spread over its entire surface. In some cases, mutant pathogenic bacteria that lack fimbriae cannot adhere to their usual target host cell surfaces, and thus cannot cause disease (Burrows, 2005).

Fimbriae are appendages composed of curlin proteins that can be found on many gram-negative and some gram-positive bacteria. They are thinner and shorter than flagella. Fimbriae are known to bind plasma proteins and initiate proteolytic cascades to activate calcium influx and signal transduction cascades in host target cells and act as invasion and motility factors (Connell *et al.*, 1996). Fimbriae are the primary mechanisms of virulence for *E. coli, Bordetella pertussis*, *Staphylococcus* sp. and *Streptococcus* sp. The presence of fimbriae greatly enhances the bacteria's ability to attach to host cells and tissues and cause disease (Telford *et al.*, 2006). The abundance of fimbriae among bacteria has motivated numerous efforts to develop classification schemes. Even though initial classification approaches used morphological criteria to classify these adhesion structures, recent studies group fimbriae by their assembly pathway and receptor specificity or antigenic variation. The major assembly classes present in gram-negative bacteria include those for conjugative fertility (F) fimbriae (F pili), type IV fimbriae (e.g., toxin-coregulated pilus), fimbriae assembled by the extracellular nucleation/precipitation pathway (curli), and fimbriae assembled by the chaperone/usher-dependent pathway (Nuccio & Baumler, 2007).

2.1.1.2 Flagella

A flagellum is a lash-like appendage that protrudes from the cell body of several bacteria, usually gram-negative. The most studied flagellate bacteria are *Escherichia coli*, *Salmonella typhimurium* and also the ulcer-causing *Helicobacter pylori*, which uses multiple flagella to propel itself through the mucus lining to reach the stomach epithelium (Lacy & Rosemore, 2001). Bacterial flagellum is a 20 nanometer-thick hollow tube, composed of the protein flagellin and consists of three parts: the basal body, the hook, and the filament (Figure 2.1).

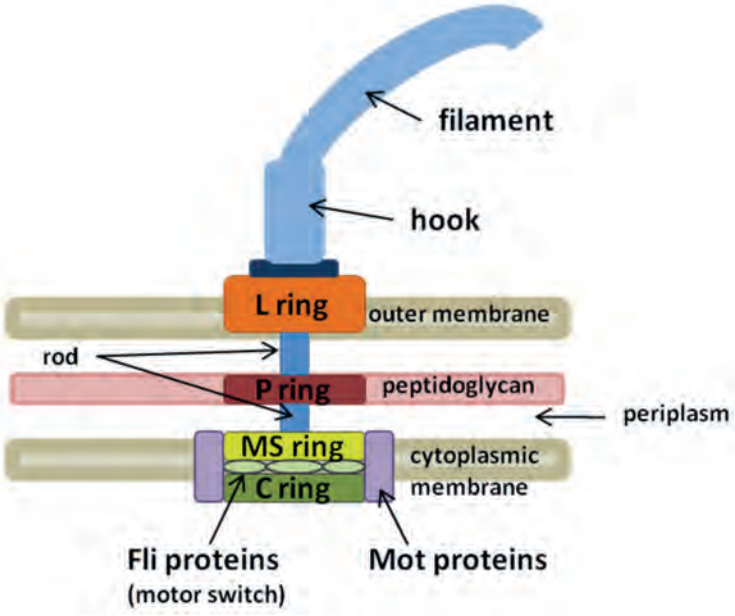

Figure 2.1: Schematic representation of the flagellar structure in gram-negative bacteria. The three parts of the flagellum are: the filament, the hook, and the basal body. The basal body contains the flagellar protein export apparatus, the MS-C ring (reversible rotor), the rod (drive shaft), and the LP rings (brushing).

Flagella are essential structures in pathogenic bacteria. Their functions are: i) provide motility, ii) promote adherence by acting as bacterial adhesins, iii) promote bacterial biofilm formation supporting pathogen survival *in vivo*, iv) translocate virulence proteins into host cells *via* special type III secretion systems and v) trigger host pro-inflammatory responses through the Toll-like receptor 5 (TLR5) signaling pathway (Duan *et al.*, 2013).

Expression of bacterial flagella has been shown to be required for maximal bacterial adherence, colonization and subsequent invasion, and could influence the virulence of many enteric pathogens. Flagellar motility benefits bacteria through the capacity to move toward favorable environment, thus enabling successful competition

with other microorganisms. Some bacterial species require motility for successful infection and flagella serves as virulence factors in several such gram-negative bacteria such as *S. typhimurium*, *Vibrio cholera*, *L. monocytogenes* and *P. aeruginosa*. Flagella proved to enhance *in vitro* bacterial invasion in *E. coli, L. monocytogenes* and *S. enteritidis*. Flagellar type III secretion system (T3SS) is associated with bacterial pathogenicity. The bacterial flagellum is a special type III secretion apparatus, which derives from a common ancestral system and shares at least nine homologous constituents with virulence-related T3SS (Duan *et al.*, 2013). These T3SS are also exporting same or similar virulence factors into the host cells, in some instances. Although it was previously thought that the flagellum export system was responsible solely for secreting proteins involved in filament biogenesis, flagellar T3SS could be an additional mechanism for the export of virulence factors by pathogens. Some of the virulence factors which have been recently proved to be secreted by the flagellar T3SS are: the anti-sigma factor FlgM, virulence-associated phospholipase of *Y. enterocolitica* (YplA), *Campylobacter* invasion antigen (Cia) proteins, and some virulence determinants from *Bacillus thuringiensis* (Duan *et al.*, 2013; Marchetti *et al.*, 2004).

Flagellum-mediated motility is not only essential for increasing pathogen-host interactions and promoting the subsequent adherence and colonization of the host cells and tissues, but also for biofilm formation.

2.1.1.3 Polysaccharides

A series of sugar-based substances, with complex structures are part of the cell wall of gram-positive and gram-negative bacteria. These molecules have an important role in bacterial pathogenesis, by promoting attachment, helping microorganisms to avoid host defense systems and being involved in maintaining of a certain electric charge of the cells. The study of these glycoconjugates lead to the development of a new research field called bacterial glycomics (Reid *et al.*, 2010).

The most common non-protein adhesins are *polysaccharides*. They compose network-based structures such as *glycocalyx* and *capsule*, and may enter within the composition of unregulated structures, such as bacterial *mucous layer*. Exopolysaccharides produce capsular structures in many pathogenic bacteria and they are considered potent virulence factors. Surface polysaccharides have mainly two roles:

i) Promote the attachment of bacteria to host cells and tissues. They intensify the formation of hydrophobic interactions between bacterial and host surface molecules. Polysaccharides promote biofilm formation, which closely attach along host tissues (Beauregard *et al.*, 2013).
ii) Protect the bacteria cells against host defense systems. Capsule inhibits phagocytosis because capsulated bacteria form microcolonies and they are difficult to be phagocytosed by the macrophages. On the other hand, the complement and antibodies have restricted access to the bacteria cell surface because of the protective capsule. Some species, such as *Streptococcus pyogenes* and *Pasteurella multocida*

develop a capsule with a chemical composition similar to the host tissues components: i.e. hyaluronic acid. This capsule "camouflages" the bacterial cell against host immune system (Singh *et al.*, 2011).

The molecular polysaccharide fibrous matrix favors bacteria cells growth and stabilizes the biofilm. The stability of biofilm insures the continuous formation of microcolonies and the persistence of chronic infection. From its structure, bacteria cells periodically detach and go in the blood and other biological tissues, thus expanding the infection.

2.1.1.4 Biofilms

Biofilms are sessile microbial communities attached to a surface by using extracellular polymeric substances and are phenotypically distinct from their planktonic counterparts (Costerton *et al.*, 1995). Microorganisms produce biofilms as a response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. After the attachment of bacterial cells through their adherence appendages, they switch to the biofilm mode of growth. A phenotypic shift in behavior occurs where multiple genes are differentially regulated. Bacterial biofilm formation occurs in a sequential process starting with: i) the transport of bacteria to a surface, ii) initial attachment, iii) formation of microcolonies, iv) biofilm maturation (Tolker-Nielsen *et al.*, 2000) and v) dissemination (Figure 2.2). Biofilms will develop on virtually every non-shedding surface in a non-sterile aqueous or environment with high humidity and are a major threat to the hospital environment. Recent estimations revealed that more than 80% of all infections are related to biofilm formation (Holban *et al.*, 2014a). Bacterial biofilms are responsible for several chronic diseases that are difficult to treat, for example: cystic fibrosis associated infections, endocarditis, cystitis, and infections associated with the presence of indwelling medical devices.

Biofilm-growing bacteria cause chronic infections that are characterized by persistent of inflammation and tissue damage, despite antibiotic therapy and the innate and adaptive immune and inflammatory responses of the host.

The high resistance and tolerance of biofilm embedded bacteria to virtually any antimicrobial is related to several factors or combinations of factors. These include restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of several resistance genes (Lewis, 2001). Biofilms are enclosed within an exopolymer matrix that can inhibit or reduce the diffusion and control the binding of antimicrobial substances. The matrix is usually composed of polysaccharides and it provides effective resistance for biofilm cells against large molecules such as antimicrobial proteins – lysozyme and host complement. This structure acts as a diffusion barrier which is also effective against smaller antimicrobial peptides, such as

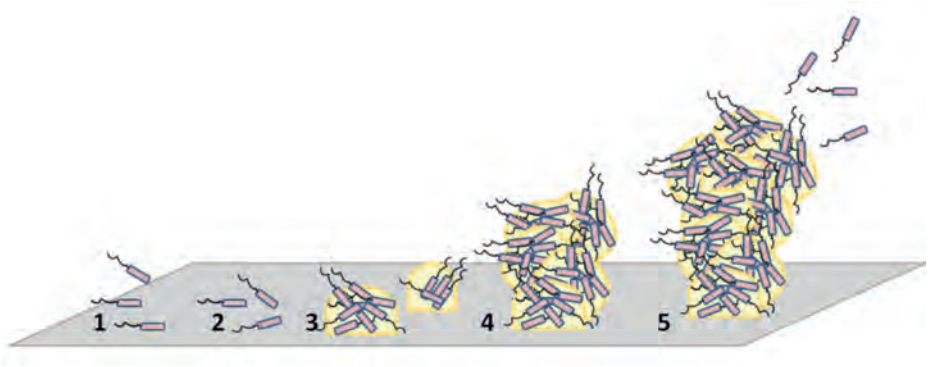

Figure 2.2: Biofilm formation and colonization of a surface. 1. Bacterial transport to the surface, 2. Initial attachment, 3. Development of microcolonies, 4. Biofilm maturation, 5. Dissemination.

defensins and their analogs and its negatively charge is very effective in protecting cells from positively charged aminoglycoside antibiotics by restricting their permeation, possibly through binding of these antibiotics (Ishida *et al.*, 1998). Decreased growth rates observed in biofilm embedded bacteria represent also an efficient resistance mechanism, since it is accepted that virtually all antimicrobials are more effective in killing rapidly growing cells. The decreased bacterial metabolic activity and increased doubling times of the bacterial cells seem to be associated with the existence of gradients of nutrients and oxygen which exist from the top to the bottom of biofilms. It is believed that dormant cells are actually responsible for some of the tolerance to antimicrobials. Some populations of bacteria in biofilms are able to differentiate into persister cells, which are considered to be non-growing or slow-growing cells and also have a greatly reduced susceptibility to antibiotics. These small subpopulations of bacteria within the biofilms that differentiate into dormant cells are able to survive extreme antibiotic treatment, which have been hypothesized to be the result of phenotypic variations rather than due to stable genetic changes (Holban *et al.*, 2013). Biofilm growth is associated with an increased level of mutations as well as with quorum-sensing-regulated mechanisms. Conventional resistance mechanisms such as chromosomal ß-lactamase, upregulated efflux pumps and mutations in antibiotic target molecules in bacteria also contribute to the survival of biofilms (Hoiby *et al.*, 2010). Furthermore, in biofilms the expression of certain genes that may confer resistance are upragulated. For example, β-galactosidase was expressed in response to imipenem and piperacillin treatment in *P. aeruginosa* biofilms. Genes encoding for multidrug resistance efflux (MDR) pumps are also upregulated in biofilms and are believed to play a greater role in biofilm resistance at low antibiotic concentrations. Recent studies indicate that some unknown MDR pumps might be over-expressed in *P. aeruginosa* biofilms (Lewis, 2001).

Biofilms that develop on medical devices represent a major threat for patients undergoing invasive exploratory investigations and in those with implanted prostheses.

These biofilms may be mono or poly-specific and may be composed of bacteria (gram-positive or gram-negative bacteria) or yeast. Bacteria commonly isolated from prosthetic devices include: *Enterococcus faecalis*, *S. aureus, S. epidermidis*, *Streptococcus viridians*, *E. coli, Klebsiella pneumoniae, Proteus mirabilis,* and *P. aeruginosa* (Donlan, 2001). Since microorganisms growing in biofilms are difficult or impossible to treat with antimicrobial agents, microbial biofilms may pose a public health problem, especially for persons requiring indwelling medical devices.

2.1.1.5 Secretion Systems

In order to interact with their host, pathogenic strains usually secrete some virulence factors, which is capable of modifying the metabolism of host cells, contributing to disease. In gram-negative bacteria this secretion process involves the crossing of both the inner and the outer membranes. Six secretion mechanisms have been described in gram-negative bacteria called type I, type II, type III, type IV, type V and type VI secretion systems.

Type I secretion systems (T1SSs) are simple, tripartite systems facilitating the passage of proteins of various sizes in one-step across the cell envelope of gram-negative bacteria. The most well investigated T1SS is the haemolysin secretion system in *E. coli*. They consist of an ATP-binding cassette (ABC) transporter or a proton-antiporter, an adaptor protein that bridges the inner membrane and outer membrane and an outer membrane pore. T1SSs are involved in the secretion of cytotoxins belonging to the RTX (repeats-in-toxin) protein family, cell surface layer proteins, proteases, lipases, bacteriocins and haem-acquisition proteins (Fronzes *et al.*, 2009).

Type II secretion systems (T2SSs) are multicomponent machines that use a two-step mechanism for translocation. During the first step, the precursor effector protein is translocated through the inner membrane by the Sec translocon or the Tat pathway (Korotkov *et al.*, 2012). Once in the periplasm, the effector protein is translocated by the T2SS through the outer membrane. The T2SS translocon consists of more than 15 proteins that are found in bacterial membranes, the cytoplasm and the periplasm. The T2SS shows an evolutionary relationship with the type IV pilus assembly machinery (Fronzes *et al.*, 2009).

Type III secretion systems (T3SSs), also called injectisomes, mediate a single-step secretion mechanism and are used by many plant and animal pathogens, including *Salmonella* spp., *Shigella* spp., *Yersinia* spp., enteropathogenic and enterohaemorrhagic *E. coli* and *P. aeruginosa*. The T3SS is best illustrated by the *S. enterica* ssp. *enterica* serovar *Typhimurium* system, which uses the invasion (Inv) and Prg proteins. T3SSs deliver effector proteins into the eukaryotic host cell cytoplasm in a Sec-independent manner. T3SSs have proved to be genetically, structurally and functionally related to bacterial flagella. They are composed of about 20 different proteins, which form a large supramolecular structure crossing the bacterial cell envelope (Journt *et al.*, 2005).

Type IV secretion systems (T4SSs) are versatile systems that are not only found in gram-negative and gram-positive bacteria, but also in cell wall-less bacteria and some archaea. Similar to the type III systems, this apparatus extends beyond the cell surface as a pilus structure that is important for direct contact with and penetration of the recipient cell surface. This secretion machinery secretes a wide range of substrates, from single proteins to protein–protein and protein–DNA complexes. These machines have broad clinical significance for not only delivering bacterial toxins or effector proteins directly into targeted host cells, but also for direct involvement in biofilm formation and the rapid horizontal spread of antibiotic resistance genes among the microbial community. The most investigated T4SS is the *Agrobacterium tumefaciens* VirB/D system (Zechner *et al.*, 2012).

Type V secretion systems (T5SSs) include autotransporters and two-partner secretion systems that translocate the substrates in two steps. Autotransporter proteins, such as NalP from *Neisseria meningitides,* are multidomain proteins that are secreted as precursor proteins across the inner membrane in a Sec-dependent process. Subsequently, the translocator domain of the protein inserts into the outer membrane and facilitates surface localization of the passenger domain. In two-partner secretion systems, a separate translocator protein mediates the secretion of the effector protein through the outer membrane. Over 700 proteins with functions that include autoaggregation, adherence, invasion, cytotoxicity, serum resistance, cell-to-cell spread and proteolysis use these two secretion systems to cross both inner and outer membranes during this simple two-step process (Henderson *et al.*, 2004).

Type VI secretion systems (T6SSs) are recently described secretion systems that are found in several pathogens such as *P. aeruginosa*, enteroaggregative *E. coli*, *S. Typhimurium*, *Vibrio cholerae* and *Yersinia pestis*. T6SSs are multi-component systems that could be composed of 12 to 25 subunits (Russell *et al.*, 2014). These complex bacterial export pathways have the capacity to translocate protein effectors into a diversity of target cell types, but their specific functions are currently unknown. Recent studies have demonstrated that this apparatus is involved in both antagonistic and non-antagonistic behaviours of several gram-negative bacteria. Structural models of the T6SS indicate that the apparatus is composed of at least two complexes, a dynamic bacteriophage-like structure and a cell-envelope-spanning membrane-associated assembly. These secretory systems are also involved in virulence modulation in certain bacteria (Fronzes *et al.*, 2009).

2.1.2 Soluble Virulence Determinants

Pathogenic bacteria are able to produce a full arsenal of soluble virulence factors to enable their ability to colonize and invade the host and also modulate the host immune defense systems to cause tissue damage. The production of all virulence factors is

strictly controlled and regulated by a cell-to-cell density dependent signaling system called Quorum Sensing (QS) (Holban & Lazar, 2011).

2.1.2.1 Proteases

The genetic flexibility of bacterial species lead to the production of a great arsenal of virulence factors involved in host invasion and in the progression of the infectious process.

Clp and Lon proteases are the major conserved proteases in the bacterial world, which directly contribute to virulence through the timely degradation of virulence regulators and indirectly by providing tolerance to adverse conditions such as those experienced in the host (Frees *et al.*, 2013). These extracellular proteases are activated in complex cascades involving auto-processing and proteolytic maturation. Lon proteases are involved in many virulence-related properties such as swarming, twitching and biofilm formation and decrease antimicrobial resistance in some bacteria species, such as *P. aeruginosa* (Breidenstein *et al.*, 2012).

Some bacteria, such as *S. pyogenes, S. aureus* and *P. aeruginosa*, produce a variety of enzymes that may cause damage to host tissues. These enzymes include *hyaluronidase*, which degrades the connective tissue component hyaluronic acid; a wide range of proteases and lipases; *DNAses*, which break down DNA, and *hemolysins* which break down a variety of host cells, including red blood cells (Keen, 2012).For example, *Helicobacter pylori* is able to survive in the acidic environment of the human stomach by producing the enzyme *urease*. Colonization of the stomach lining by this bacterium can lead to Gastric ulcer and cancer. The virulence of various strains of *H. pylori* tends to correlate with the level of production of urease, which is a potent immunogen that elicits a vigorous immune response (Eaton *et al.*, 1991). Also, urease has been demonstrated as a potent virulence factor for some species, including *Proteus mirabilis* and *Staphylococcus saprophyticus* (Mobley, 2001).

There are several virulence factors which facilitate bacterial invasion of a host by disrupting host cell membranes. This disruption allows for transport across epithelial layers of tissue and skin. The *internalin* surface proteins found on *Listeria monocytogenes* allow them to invade mammalian epithelial cells *via* transmembrane proteins. *L. monocytogenes* have evolved two major molecular invasion proteins also known as invasins: Internalin A (InlA) and Internalin B (InlB), which promote internalization into nonphagocytic cells where this bacterium can grow in the cytosol as a facultative intracellular pathogen and directly spread to neighboring cells through actin-based motility. Recent studies have reported that *L. monocytogenes* uses InlA protein to invade the tips of the intestinal villi, a location at which cell extrusion generates a transient defect in epithelial polarity exposing E-cadherin, the receptor for InlA, on the cell surface (Pentecost *et al.*, 2010).

Proteases are well investigated in the opportunistic pathogen *P.aeruginosa*. Proteases produced by this bacterium may also be found within the host. For example, in patients with cystic fibrosis (CF), *P. aeruginosa* proteases such as elastase B, alkaline

protaease, protease IV and PasP have been detected in the lungs. Elastase is one of the prototype virulence factors of *P. aeruginosa* regulated by the quorum sensing cascade. *Elastase B* is a metalloprotease belonging to the M4 thermolysin peptidase family, which is encoded by the *lasB* gene. Histologic studies have detected abnormal elastin fibers in lung alveoli of CF patients, but elastin can also be found around vascular tissue in the external elastic laminea and its disintegration seems to be associated with vasculitis during *P. aeruginosa* infection (Schmidtchen *et al.*, 2003).

Elastase B can also interact with proteins of the human immune defense system and degrade immunoglobuline A (IgA) and G (IgG) (Hoge *et al.*, 2010).

Staphylolysin, also known as *elastase A* (LasA), is one of the most abundant endopeptidases secreted by *P. aeruginosa*. LasA has low elastolytic activity but high staphylolytic activity and cleaves a wider range of glycine-containing proteins, including tropoelastin-derived pentapeptides, glycine-rich synthetic peptides and specific sequences present in elastin. LasA enhances the activity of LasB in the establishment and progression of *P. aeruginosa* infection. Moreover, LasA enhances the activity of several host elastolytic proteases, including human leukocyte elastase and human neutrophil elastase (Azghani *et al.*, 2002).

Protease IV, also known as lysyl endopeptidase or iron-regulated protein PrpL, was first identified and characterized as a 26 kDa serine protease present in the culture supernatant of *P. aeruginosa*. This protease is able to bind and cleave fibrinogen and plasminogen and is able to degrade several surfactant proteins such as SP-A, SP-D and SP-B. Recent research on the impact protease IV in bacterial virulence using *in vivo* murine models has revealed that this protease plays an important role in the development of keratitis of the cornea (Caballero *et al.*, 2004).

Alkaline protease (AprA), also known as aeruginolysin, is a member of the so-called metzincin superfamily and is one of the secreted zinc-dependent metalloendopeptidase by *P. aeruginosa*. AprA is capable of cleaving a large number of physiological substrates *in vitro* such as laminin and has a direct function in the invasion and hemorrhagic tissue necrosis in infections caused by *P. aeruginosa*. Along with elastase B, AprA can inactivate human γ-interferon and human tumor necrosis factor-α and also inhibit the function of neutrophils by interfering with chemotaxis. AprA is able to inactivate different human protease inhibitors such as serpins, the C1-inhibitor and α-1-antichymotrypsin, IgG, epithelial neutrophil activating protein-78 (ENA-78) and monocyte chemotactic protein 1 (MCP-1). Moreover, this protease is capable of interfering with human lymphocyte function, presumably due to on the degradation of IL-2 and IL-6 (Leidal et al., 2003).

Recent studies have revealed that secreted bacterial proteases are able to control bacterial virulence repertoire and are involved in the progression of several diseases. For example, exoproteome analyses has revealed that Sae-regulated protease *aureolysin* is a major determinant of the *S. aureus* secretome and several phenol-soluble modulins such as aureolysin-degraded, osteolytic peptides may trigger osteoblast cell death and bone destruction. Further studies using established murine models for

pathogen-induced bone remodeling, define Sae as critical for osteomyelitis pathogenesis, and identify protease-dependent exoproteome remodeling as a major determinant of the staphylococcal virulence repertoire (Cassat *et al.*, 2013).

2.1.2.2 Toxins

Toxins are a major group of virulence factors and are divided into two groups: *endotoxins* and *exotoxins*.

Endotoxins are represented by components of the cell wall of gram-negative bacteria, namely lipopolysaccharides (LPS). The lipid A, located at the very tip of the lipid tail of the LPS structure, is actually toxic. Lipid A is also a very potent antigen and stimulates an intense host immune response (Levinson, 2010). These molecules are named endotoxins because they are never released from an intact bacteria cell. The innate immune system is able to recognize a broad range of pathogens associated molecular patterns (PAMPs), including LPS. This ability is mediated by Toll-like receptors (TLR), which are a series of receptors able to detect a variety of pathogen epitopes. TLR-4 is responsible for recognizing LPS. Lipid A from the structure of LPS stimulates the release of immune response cytokines and it may cause fever and other symptoms observed during gram-negative infections. High amount of LPS may lead to septic shock (or endotoxic shock) and in severe cases, can cause death (Levinson, 2010).

Exotoxins are actively secreted by some bacteria and can may a wide range of effects including the inhibition of certain biochemical pathways in the host. Depending on their cellular site of action, exotoxins can be divided into three main groups:

- Type-I toxins – membrane acting (e.g. super antigens or heat stable enterotoxins, where the typical method of toxicity is to bind to cell surface receptors and trigger intracellular signaling pathways),
- Type-II toxins – membrane damaging (e.g. channel forming toxins or enzymatically active toxins, where the typical method of toxicity is cell lysis, modulation of signal transduction or enzymatic action),
- Type-III toxins – intracellular (e.g. bacteria specific toxins such as cholera toxin, pertussis toxin, diphtheria toxin etc., where the typical method of toxicity varies: inhibition of protein synthesis, modulation of host cell signaling, inhibition of neurotransmitters etc.) (Lemonnier *et al.*, 2007).

Exotoxins are usually produced from cells growing in wound or on mucosal surfaces and may also be acquired directly from ingestion (e.g. contaminated food).

The tetanus toxin (tetanospasmin) secreted by *Clostridium tetani* and the botulinum toxin secreted by *Clostridium botulinum* are the two most potent known exotoxins. Exotoxins with severe impact on the host cells and tissues are produced by a range of gram-positive and gram-negative bacteria species including *Bacillus anthracis*, *E. coli*, *Vibrio cholera*, *Clostridium perfringens* and *Clostridium difficile* (Lebrun *et al.*, 2009).

2.1.2.3 Quorum Sensing

Quorum sensing (QS) represents the microbial regulation of gene expression in response to fluctuations in cell-population density. This is achieved by producing and releasing chemical signal molecules called autoinducers (AIs) that increase in concentration depending on the cell density. Both gram-positive and gram-negative bacteria use QS communication circuits to regulate a diverse array of physiological and pathogenesis-related activities (Miller & Bassler, 2001). These signal-dependent regulatory mechanisms control all known bacterial processes, however, the most investigated are: symbiosis, virulence, competence, conjugation, antibiotic production, motility, sporulation, and biofilm formation (Miller & Bassler, 2001). Cell-cell communication *via* AIs occurs both within and between bacterial species and also between pathogens and their hosts (Holban *et al*., 2014b; Miller & Bassler, 2001).

There are three main and widely accepted types of QS signaling systems in bacteria, but recently a forth system has been described. The QS regulatory systems are:

- The LuxR/I-type systems, primarily used by gram-negative bacteria, in which the signaling molecule is usually an acyl-homoserine lactone (AHL),
- The peptide signaling systems used primarily by gram-positive bacteria,
- The luxS/AI-2 signaling used for interspecies communication,
- The AI-3/epinephrine/norepinephrine interkingdom signaling system (Reading & Sperandio, 2006).

The LuxR/I signaling system was first described in *Vibrio fischeri* to regulate the bioluminescence production. This signaling system is regulated by two proteins, LuxI, which is responsible for the production of the AHL autoinducer, and LuxR, which is activated by this autoinducer to increase transcription of the target genes/operon. Many homologs of Vibrio LuxR–LuxI have been identified in several bacteria species and in all cases the bacteria produce an AHL autoinducer, which binds to the LuxR protein and regulates the transcription of several genes involved in a variety of phenotypes. These include the production of antibiotics in *Erwinia* sp., motility in *Yersinia pseudotuberculosis*, pathogenesis and biofilm formation in many bacterial species such as *P. aeruginosa*, *E. coli*, and *Salmonella* sp. (Holban & Lazar, 2011).

The LuxI-type proteins are represented by the AHL synthases and they are characterized by a conserved homoserine lactone ring connected through an amide bond to a variable acyl chain. On the other hand, the LuxR-type proteins are transcription factors, which, upon binding to the AHL signal, regulate transcription of their target genes. Even though LuxR-type proteins usually recognize a specific AHL, most of them may also recognize unrelated AHLs due to them being involved in interspecies signaling. One of the best characterized LuxR/I-type QS systems is the one found in *P. aeruginosa* which is utilized to activate several genes involved in colonization and persistence within the host (Parsek & Greenberg, 2000). In this bacterium, QS controls production of an array of virulence factors (i.e. elastase, exotoxin A, piocianin etc.) and biofilm development. When these QS are disrupted experimentally, to significant reduction in

P. aeruginosa virulence was observed in plants and animals and inhibition of biofilm formation. The very complex and hierarchical QS system of *P. aeruginosa* depends on the production of two AHLs: N-(3-oxododecanoyl)-l-homoserine lactone (3OC12-HSL) and N-butanoyl-l-homoserine lactone (C4-HSL). These AHLs bind to and activate LasR and RlhR transcription factors: LasR complexed with 3OC12-HSL activates the transcription of *rhlR* and *rlhI* (Parsek & Greenberg, 2000).

Some bacteria species such as *E. coli* and *Salmonella* sp. have a LuxR homolog, (called SdiA), but do not have a *luxI* gene, and do not produce AHLs (Reading & Sperandio, 2006).

The peptide signaling systems is used by gram-positive bacteria and relies on the autoinduction by small peptides which, interacts with two-component systems that regulate gene transcription. These small molecules are usually products of oligopeptides that are cleaved or further modified before being exported from the bacterium by transporters. When these signaling peptides reach threshold concentrations, they are recognized by specific sensor kinases that initiate phospho-transfer to a response regulator. The most investigated peptide based QS signaling systems is the one discovered in the gram-positive species *S. aureus*. This system is called *the accessory gene regulator* (Agr) and it regulates toxin and protease secretion in staphylococci. At low cell density, the bacteria express proteins required for attachment and colonization and as the cell density becomes higher, this expression profile switches to express proteins involved in toxin and protease secretion (Reading & Sperandio, 2006). The *S. aureus* autoinducing peptide (AIP) is encoded by the *agrD* gene, which controls the activity of a protein that adds a thiolactone ring to this peptide and transports the AIP out of the cell. The AIP will then bind to its receptor, sensor kinase ArgC and ArgC's cognate response regulator, ArgA. Upon AIP binding to ArgC, ArgC transfers a phosphate to ArgA, which activates transcription of the *arg* operon for autoregulation and in addition activates transcription of the RNAIII, a regulatory RNA, which in turn leads to the repressed expression of cell adhesion factors and induces expression of secreted factors (Novick, 2003).

The LuxS/AI-2 signaling system constitutes a mix between components of gram-positive and gram-negative systems and it was investigated in *Vibrio harveyi*. This bacterium has two QS systems: i) system 1 in which the autoinducer (AI-1) is an AHL, and is primarily involved in intraspecies signaling and ii) system 2, in which the autoinducer is a furanosyl borate diester involved in interspecies signaling (Chen *et al.*, 2002). *V. harveyi* have two hybrid sensor kinases, LuxN (which sense AI-1) and LuxQ (which sense AI-2). In the absence of a signal, these proteins are intrinsic kinases and phosphorylate a complex phosphorelay system with certain enhancer-binding proteins, LuxU and LuxO, as intermediaries. Phosphorylated LuxO activates the transcription of small regulatory RNAs, which destabilize the message of the LuxR protein, which in turn can no longer activate transcription of the luciferase operon. Upon interaction with their cognate autoinducers, these sensors behave as phosphatases and the system is dephosphorylated, allowing LuxR to activate bioluminescence (Lenz *et al.*,

2004). AI-2 is synthesized by the LuxS enzyme which converts S-ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD). DPD is a very unstable compound that reacts with water and cyclizes to form several furanones, one of which is thought to be the precursor of AI-2. Several bacterial species harbor the *luxS* gene, and have AI-2 activity as measured using a *V. harveyi* bioluminescence assay. However, the genes shown to be regulated by AI-2 in other species encode for an ABC transporter in *S. typhimurium* called Lsr (LuxS-regulated) and is responsible for the AI-2 uptake. This ABC transporter is also present in *E. coli* and shares homology with sugar transporters (Reading & Sperandio, 2006).

The AI-3/epinephrine/norepinephrine signaling system was first discovered by serendipity as being associated with the LuxS system, which seems not to be devoted solely to AI-2 production. Even though LuxS seems to influence the function of AI-3, it is not involved in the synthesis of this AI *per s*e. Even though the structural analysis of AI-3 was not yet published, it seems that this signaling molecule is an aromatic compound and does not contain a sugar skeleton like AI-2 (Reading & Sperandio, 2006). It has recently been shown that the microbial intestinal microbiota and also several intestinal pathogens such as enteropathogenic *E. coli* strains from serogroups O26:H11 and O111ac:H9, *Shigella* sp., and *Salmonella s*p. produce AI-3 (Sperandio *et al.*, 2003). Besides being used in bacterial interspecies signaling, AI-3 seems to have an intrinsic role in interkingdom communication, since AI-3 crosses signals with the eukaryotic hormones epinephrine/norepinephrine in an agonistic fashion. Studies have revealed that enterohemorrhagic *E. coli* senses AI-3, produced by the microbiota and epinephrine/norepinephrine produced by the host to activate the expression of the LEE pathogenicity genes and the flagella regulon (Sperandio *et al.*, 2003). The AI-3/epinephrine/norepinephrine signaling cascade is present in several bacterial species such as *Shigella*, *Salmonella*, *Erwinia carotovora*, *Pasteurella multocida*, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*, *Chromobacterium violaceum*, *Coxiella burnetti*, *Yersinia*, *Francisella tularensis* and *Ralstonia solacearum*, suggesting that this interkingdom crosssignaling is not restricted to *E. coli* (Reading & Sperandio, 2006).

The modulation of all the cell-attached, soluble and regulatory virulence factors and many yet insufficiently elucidated virulence determinants represents an alternative approach for infection control. Many current studies are focused on efficiently implementing virulence modulation as new anti-infectious measures.

3 Alternative Methods for Infection Control

The concern that humankind is reentering the "preantibiotics" era has become very real and the development of alternate antiinfection modalities has become one of the highest priorities of modern medicine and biotechnology. The fact that alternative anti-pathogenic strategies are urgently needed is widely accepted by all clinicians and researchers in the field. The true challenge is in finding the best option in order to limit resistance and reduce possible side-effects. Recent trend in antiinfectious therapy relies in limiting the use of antibiotics because of their great toxicity and the potential of selecting resistant strains. Methods based on using natural alternative antimicrobial approaches are currently being investigated.

3.1 Anti-infectious Approaches Based on Biological Factors

3.1.1 Bacteriophages

As natural killers of bacteria, phages are obvious candidates for exploitation as anti-bacterial agents. Bacteriophages have many intrinsic characteristics which make them attractive candidates for such applications: i) they cannot replicate in eukaryotic cells or incorporate their DNA into the genome of such cells, ii) they are highly specific in their bactericidal potential, and iii) they generally target a single bacterial species and some phages are even strain specific.

The discovery of bacteriophages is generally attributed to Twort (Twort, 1936) and d'Herelle (Dublanchet & Fruciano, 2008) in the early 20th century. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented or treated by the administration of bacteriophages. Bacteriophage therapy for bacterial infections is a concept with an extensive but controversial history. A range of commercial products were distributed by companies in France (Laboratoire de Bacteriophage), Germany (Antipol), the UK (Medico-Biological Laboratories), and the US (Hausler, 2006). However, mixed therapeutic results, poor understanding of phage biology and the advent of broad-spectrum antibiotics led to the decline of phage therapy in the Western world. In the meantime, phage therapy continued to be studied and used in Eastern Europe and the Soviet Union (Lu & Koeris, 2011).

In vivo experimental data on phage therapy suggested, that phage therapy is very efficient in treating infections in laboratory animals. A recent study shows that phages may be successfully used to treat experimental *E. coli* infections in mice and reduces, by many orders of magnitude, the number of target bacteria in the alimentary tract of calves, lambs, and piglets infected with a diarrhea-causing *E. coli* strain. Phages may be also used in preventing and treating experimental disease in mice and guinea pigs infected with *P. aeruginosa* and *Acinetobacter* and studies suggested that phages

might be efficacious in preventing infections of skin grafts used to treat burn patients (Sulakvelidze *et al.*, 2001). Wright *et al.* (2009) reported improved outcomes and decreased *Pseudomonas* loads in adult patients with chronic otitis externa treated topically with bacteriophages. They also showed that successful bacteriophage therapy can be achieved if susceptible host bacteria are present at the site of application and emphasized the importance of accurately identifying bacterial infections before phage treatment (Wright *et al.*, 2009).

Phages have been shown to influence some bacterial virulence factors, such as bacterial adhesion, colonization, invasion, toxins production and spread through human tissues; resistance to immune defenses; exotoxin production; sensitivity to antibiotics; and transmissibility among humans. Phage therapy has been shown to interfere with adherence and invasion of *Streptococcus mitis* and *Salmonella enterica serovar Typhimurium*, mainly by inhibiting these phenotypes through molecular mechanisms (Wagner & Waldor, 2002). Phages may enhance resistance to serum and phagocytes in some strains of *S. aureus* and *Salmonella* sp.

Recent research demonstrated that phages might encode enzybiotics (enzymes which can be utilised as bacteriocidal agents), such as autolysins and lysozymes. These discoveries lead to the creation of databases such as phiBiotics and EnzyBase which provide data on the growing number of characterised enzybiotics (Hojckova *et al.*, 2013). Studies revealed that endolysins may be applied exogenously to specific gram-positive pathogens and they cause rapid lysis of bacteria cells. Currently phage lysins are being investigated as novel antibacterials with potential applications in healthcare, veterinary, agriculture, food and biotechnology sectors. One of the advantages of using phage lysins over antibiotics is their targeted specificity, which greatly reduces the risk of dysbiosis. Furthermore, unlike antibiotics and whole phages there have been no reports of development of bacterial resistance to phage lysins (Keary *et al.*, 2013). Phages can also stimulate virulence by encoding bacterial exotoxins. Phage-encoded exotoxins work by a variety of mechanisms and were demonstrated to be very important for *Vibrio cholerae*, *Corinebacterium diphtheriae*, and *Clostridium botulinum*. Even though no examples of phage-encoded resistance genes are known, studies suggest that phages may play an important role, *via* transduction, in the mobility of these resistance plasmids among staphylococci and streptococci (Caparon, 2000). Many prophages may be induced by environmental conditions and this lead to bacterial DNA damage and in the case of *E. coli*, activation of a molecular regulator (RecA), which in turn catalyzes the cleavage of phage repressors. Many antibiotics commonly used to treat diarrhea, for example, are known to induce Shiga toxins (Stx)-encoding phages and therefore to promote toxin production by Enterohaemorrhagic *Escherichia coli* (EHEC). Perhaps not coincidentally, numerous epidemiological studies have detected an association between increased severity of EHEC infection and treatment with antibiotics (Wagner & Waldor, 2002; Wong *et al.*, 2000).

Current challenges in using phage therapy include skepticism about the rigor of prior phage therapy studies, strict regulatory constraints placed on new clinical

therapeutics such as phages, limited phage host ranges, the evolution of bacterial resistance to phages, manufacturing challenges, systemic side effects of phage therapy and delivery.

3.1.2 Synthetic Biology

Synthetic biology is an innovative and emerging field, representing a confluence of science and engineering, aiming to (re)design biological parts, devices and systems while applying engineering principles for useful applications. Advances in this field are expected to provide valuable solutions to some urgent issues in healthcare, particularly to combat infections caused by multidrug resistant bacteria by investigating resistance mechanisms, discovering new mechanisms to develop antibiotics and develop new infection control strategies. Synthetic biology was recently used to synthesize particular phages specific for certain microbial strains in order to incorporate the circuits of interest into the engineered phages. Lytic bacteriophages were also constructed to express *dispersin B*, which can degrade the extracellular polymeric substances of bacterial biofilm. Engineered bacteriophages can act as antibiotic adjuvants to enhance the efficacy of existing antibiotics, for example, phages were designed to target the bacterial DNA damage system (SOS networks), the non-SOS networks and multiple factors related with biofilm formation, antibiotic tolerance and antibiotic penetration for ofloxacin in *E. coli* (Ruder *et al.*, 2011). Another approach relies on engineering microbes to sense and destroy a targeted pathogen. Recently an engineered *E. coli* was constructed and equipped with a synthetic genetic system comprising the quorum sensing, killing, and lysing devices, which can detect and destroy pathogenic *P. aeruginosa* through the production and release of pyocin (Saeidi *et al.*, 2011). Synthetic biology has a great impact on the design of vaccines against infectious disease. Even though, most of the vaccine research is focused on viruses, applying these approaches to develop vaccines to antibiotic resistant pathogens will be a future research topic to be explored (e.g., vaccines to MRSA, multidrug resistant *M. tuberculosis*, *Clostridium difficile*, Shiga toxin-producing *E. coli* etc.) (Pei, 2013).

3.2 Anti-infectious Approaches Based on Physical Factors

3.2.1 Cold Plasmas

Plasmas are known as the fourth state of matter after solids, liquids and gases and are formed when high-energy processes strip atoms of their electrons to produce ionized gas flows at high temperature. They have an increasing number of technical and medical applications (wound healing, blood coagulation, skin regeneration, tooth

bleaching and apoptosis of cancer cells) and hot plasmas are already used to disinfect surgical instruments (Yardimci & Setlow, 2010). The development of cold plasmas with temperatures of 35-40 °C makes this technology an attractive option for treating infections caused by resistant pathogens.

Recently, a team of Russian and German researchers showed that the treatment with low-temperature plasma was able to kill drug-resistant bacteria causing wound infections in rats and increased the rate of wound healing. A low-temperature plasma torch proved to be efficient against resistant and virulent *P. aeruginosa* and *S. aureus* bacterial species (Alkawareek *et al.*, 2012; Ermolaeva *et al.*, 2011). Cold plasmas produce large quantities of charged particles (positive and negative ion and electrons), chemically reactive species, UV radiation and electromagnetic fields. The diversity and the small size of these active agents are believed to target multiple cellular components and metabolic processes in microorganisms and therefore reduce the changes of emergence of resistance mechanisms. Although the exact mechanisms driving plasma-mediated bacterial killing are not yet well understood, it seems that plasma damages microbial DNA and surface structures in both planktonic and biofilm embedded cells, without being harmful to human tissues (Yue *et al.*, 2008). Results demonstrate that plasma is effective against pathogenic bacteria with multiple-antibiotic resistance not just *in vitro* but also in actual infected wounds. The findings suggest that cold plasmas might be a promising method to treat chronic wound infections where other approaches fail. The main advantages of plasma therapy are: i) simple design, relatively low capital and operational cost, ii) utilization of non-toxic gases, iii) operating at gas temperatures at or near room temperature, iv) non-specific action, meaning it is much harder for bacteria to develop resistance; v) it is a contact free, painless method and vi) does not contribute to chemical contamination of the environment (Ermolaeva *et al.*, 2011).

3.2.2 Photodynamic Antimicrobial Chemotherapy

Photodynamic antimicrobial chemotherapy (PACT) is a recently investigated field showing promising prospects as an alternative to antibiotic treatment in view of increasing bacterial resistance to antibiotics. This technique relies on excitation of low toxic components – photosensitizers by visible light followed by the energy transfer from the light-activated photosensitizers to molecular oxygen, which results in the production of reactive oxygen species that in turn cause irreversible damage to bacterial cellular components (Yano *et al.*, 2011). Photosensitized compounds belong to a wide spectrum of groups, such as porphyrins, phenothiaziniums, phthalocyanines, hypocrellin derivatives, squaraine derivatives (squaraine dyes, squaric acid derivatives), boron dipyrromethene derivatives, and chlorine derivatives. PACT has been extensively studied as a strategy against both gram-positive (extensively studied in *S. aureus*) and gram-negative bacteria (extensively studied in *E. coli*), including antibiotic-resistant

species and was found to be very effective especially for the control of oral and other localized infections (Tavares *et al.*, 2010). No reports have shown the development of bacterial resistance to photosensitized compounds. Despite its efficiency on localized external infections, the major disadvantage of PACT is limited tissue penetration of external light, which makes this method inappropriate for the treatment of internal body tissues. However, alternative means of photosensitizers activation, such as chemiluminescent photodynamic antimicrobial therapy (CPAT) and sonodynamic therapy (SDT) have recently been proposed. CPAT replaces the external light source by chemiluminescent light emitted during the course of a chemical reaction, while SDT is based on ultrasound-induced cytotoxicity of bioactive compounds called sonosensitizers. Both methods have shown a great antimicrobial activity against the gram-negative *E. coli* and gram-positive *S. aureus* (both methicillin-sensitive and methicillin-resistant strains) (Nakonechny *et al.*, 2013).

3.3 Chemical Virulence Modulators and Alternative Antimicrobial Compounds

Although a wide selection of strategies are aimed at targeting virulence factors and the genes encoding them, a special attention was given to natural and synthetic small molecules able to inhibit virulence factors and virulence factor expression (Hung *et al.*, 2005). A series of compounds widely found in biological systems such as alkaloids, flavonoids and peptides have shown antimicrobial effect or their ability t regulate virulence. To better understand key virulence factors in hopes that new diagnostic techniques, specific antimicrobial compounds, and effective vaccines or toxoids may eventually be produced to treat and prevent infections, a series of methods have emerged. Three general experimental approaches are used to identify virulence: biochemically, immunologically, and genetic investigations (Cushnie & Lamb, 2011). However, because of bacteria versatility and virulence factors diversity, these approaches may vary depending on the strain and compound-related particularities, but also by the purpose of the investigation.

3.3.1 Natural Virulence Modulators

Natural compounds are preferred in most biomedical applications because of their high efficiency and biodegradability, making them perfect candidates for ecological anti-infectious strategies. A wide range of natural eukaryotic (Li *et al.*, 2009a; Teplitski *et al.*, 2004) and prokaryotic (Ditu *et al.*, 2011) – derived compounds may be efficient in modulating microbial virulence and interfering with bacterial cell-to-cell communication (Hentzer *et al.*, 2003). Recent technological progress makes available a robust

group of methods and techniques to investigate the biological applications of many naturally occurring compounds on virulence modulation and development of alternative antimicrobials (LaSarre & Federle, 2013).

3.3.1.1 Bacterial-derived Virulence Modulators

The normal human microbiota is composed by more than 1000 species and it is estimated that there are 10 times as many bacterial cells within the gastrointestinal tract as there are human cells within our bodies (Holban *et al.*, 2013). The normal microbiota plays an essential role in mammalian nutrition, physiology, development, immunity and behavior, therefore disruption of this structure and balance of this community leads to dysbiosis and disease. Furthermore, competition between resident microorganisms and pathogens is essential for the health-disease balance and it is influenced by the expression of virulence factors by pathogens and by the nutritional requirements of both populations (Kamada *et al.*, 2012). Recent studies reveal the importance of bacteria cell-to-cell QS signaling, by highlighting the benefits of density-dependent fitness, bacteria density being crucial in infections. These dynamics can steer the survival, colonization, and clearance of pathogens in the gut and all these findings lead to the development of a novel antiinfectious approach, by using probiotics. *Probiotics* are live, nonpathogenic bacteria that contribute to the health and balance of the intestinal tract. Probiotic bacteria have been documented as being effective in biotherapeutic applications against gastrointestinal pathogens like, *Helicobacter pylori, Salmonella, Escherichia coli, Listeria monocytogenes*, and rotaviruses. This alternative therapeutic application of probiotics to protect against gastrointestinal pathogenic infections may be of great importance for future medicinal use (Thirabunyanon, 2011).

After entering the host gut, pathogens have to face hostile condition in order to survive and multiply. Excepting host defense mechanisms, commensal bacteria are another challenge pathogens have to face. During the early stages of infection, pathogens produce many virulence factors in order to be able to adhere, colonize and multiply within the host.

Very recent studies report that virulence factors produced by pathogens also serve as a competitive advantage against high numbers of commensals (Sperandio, 2012). On the other hand, commensals fight back limiting nutritional resources and interfering with pathogens communication and virulence, finally leading to pathogen clearance during late infection. Bacteria composing normal microbiota are vital for pathogen clearance, since it have been shown that axenic animals are unable to clear infections, even if low dose of pathogens are used (Holban *et al.*, 2013).

Although the specific molecular mechanisms are widely unknown, many compounds produced by commensal bacteria and probiotics have proved to be effective against pathogenic microorganisms. For example, subinhibitory concentrations of phenyl lactic acid, produced by *Lactobacillus* sp. probiotic strains significantly attenuates the virulence and pathogenicity of many *P. aeruginosa* and *S. aureus* susceptible

and resistant clinical isolates. It is also believed that this compound acts as a QS modulator molecule (Chifiriuc *et al.*, 2009). Furthermore, it seems that *L. acidophilus* produce a set of yet unrevealed molecules that act as QS inhibitors and directly interacts with bacterial transcriptional regulators responsible for the transcription control of *E. coli EHEC O157* genes involved in colonization. Same authors also demonstrate that this probiotic strain significantly modulates the production of AI-2 signaling molecule in *E. coli*, the expression of important virulence-related genes and production of Shiga toxin (Medellin-Peña *et al.*, 2007).

Recent studies show that bacterial-derived uracil acts as a ligand for dual oxidase (DUOX)-dependent reactive oxygen species generation in the gut of the model organism *Drosophila melanogaster* and that uracil production in bacteria causes inflammation of the gut, controlling also the balance between the coordination of an efficient antimicrobial system aiming to eliminate pathogens while tolerating symbiotic commensal microbiota. However, the molecular mechanisms controlling this process are only partially understood. It seems that acute and controlled uracil-induced immune response is required for efficient elimination of bacteria, intestinal cell repair and host survival during infection of pathogenic species. Among resident gut microbiota, uracil production is absent in symbionts, allowing harmonious colonization without DUOX activation, whereas uracil release from opportunistic pathobionts provokes chronic inflammation. These results reveal that bacteria with distinct abilities to activate uracil-induced gut inflammation, in terms of intensity and duration, act as critical factors that determine homeostasis or pathogenesis in gut-microbe interactions (Lee *et al.*, 2013).

Another study demonstrates that a soil-dwelling species of *Bacillus* produces a lactonase (AiiA), is able to hydrolyze the homoserine lactone ring of all known AHLs molecules. *In vivo* experiments showed that when transgenically expressed in the QS plant pathogen, *Erwinia carotovora*, the bacteria had greatly attenuated virulence and caused only minor soft rot symptoms, compared with wild-type Erwinia. Various AHL lactonases not belonging to the AiiA clade have also been identified. Metagenomic analyses facilitated the discovery of QlcA, BipB01, BipB04, BipB05, and BipB07, and screens for AHL-degrading bacteria leading to the identification of QsdA of *Rhodococcus erythropolis* strain W2, AiiM of *Microbacterium testaceum*, AidH of *Ochrobactrium* sp. strain T63, and QsdH of *Pseudoalteromonas byunsanensis* strain 1A01261 (LaSarre & Federle, 2013). Many data support the overall value of these lactonases in quorum quenching and disease prevention.

3.3.1.2 Fungal-derived Virulence Modulators

Prokaryotes often coexist and interact with different species of eukaryotes. Bacteria and fungi generally interrelate in a variety of niches like soil, animals, food etc. The relationships between bacteria and fungi are well known; however, it is often

underestimated how intimate and decisive these interactions really are for the behavior and survival of each participant.

Certain fungal compounds can be effective in modulating QS signaling and virulence in some pathogenic bacteria. Rasmussen and his collaborators have shown that certain compounds produced by *Penicillium* sp., as patulin and penicillic acid have an inhibitory role on QS molecules, affecting the expression of 45-60% genes regulated by QS in certain bacteria (Rasmussen *et al.*, 2005). Mycorrhized and nonmycorrhized fungal species, belonging to Ascomycota and Basidiomycota phylums, have the capacity to hydrolyze *P. aeruginosa* AHLs through a lactonase activity, but the responsible molecules remain unknown. Farnesol, a signaling molecule involved in inducing the transition from hyphal to the yeast state in *C. albicans* can alter the production of toxic phenazines, like pyocyanin, in *P. aeruginosa*. Moreover, farnesol may generate oxygen reactive species in a great number of microbial species (Morales & Hogan, 2010). This process seems to play an important role in the competition between bacteria and fungi and may have a significant impact on host response and the infectious process (Holban *et al.*, 2013).

3.3.1.3 Algae-derived Virulence Modulators

Many photosynthetic algae taxons have been proved to interfere with bacteria virulence, cell-to-cell communication and pathogenesis.

The unicellular soil-freshwater algae species *Chlamydomonas reinhardtii* and *Chlorella* sp. may secrete substances that mimic the activity of AHL signal molecules used by many bacteria for QS regulation of gene expression involved in virulence control (Teplitski *et al.*, 2004). Although the specific molecular interaction is not yet known, studies showed that some algae proteins that are affected by bacteria infections, as chaperonins, nitrogen regulatory protein PII, and GTP-binding proteins are involved in this bacteria modulation (Holban *et al.*, 2013). Some algal – derived AHL – related compounds proved to cancel the stimulatory effects of *E. coli, P. aeruginosa* and *V. fisheri* AHLs on the accumulation of seven of these proteins, providing evidence that the secretion of AHL mimics by the alga could be effective in disruption of QS in naturally encountered bacteria (Teplitski *et al.*, 2004).

The most well investigated algal – derived molecules able to modulate bacteria QS signaling and virulence are furanones and their derivatives. Brominated furanones are among the first recognized small-molecule inhibitors of quorum sensing. However, because of their toxicity, commercial or therapeutic use of furanones is restricted. They are used as helpful molecular probes in understanding signaling and the consequences of inhibition (LaSarre & Federle, 2013).

The algae *Delisea pulchra* produces molecules with a broad-spectrum antimicrobial activity targeting both gram-negative and gram-positive bacteria. *D. pulchra* – derived furanones interfere with AHL recognition systems and inhibit some virulence phenotypes as swarming motility in *Serratia liquefaciens*. This effect seems to

be mainly due to a competitive interaction between AHLs and furanones for the LuxR-type receptor protein (Givskov *et al.*, 1996).

Furanones also interfere with the bioluminescence in *V. harveyi*, disrupting QS regulated gene expression, even when used in micromolar concentrations (Defoirdt *et al.*, 2007). In the opportunistic pathogen *P. aeruginosa*, furanone significantly reduces and even disrupt gene expression of some key pseudomonadal virulence gene expression. Reduced activities of exoprotease, pyoverdin, and chitinase, and disruption of biofilms have been observed after addition of low concentrations of furanones. Moreover, *in vivo* studies revealed that furanones remain active in biological systems, decreasing mortality related with virulent *P. aeruginosa* strains of the infected mice (Hentzer *et al.*, 2003).

Even though furanone signaling in bacteria is far from being understood at molecular level, recent studies suggest that the AI-2 pathway may be involved in this cross-signaling between bacteria and algae. This hypothesis is supported by studies demonstrating that chemotaxis and flagellar biosynthesis genes in *E. coli* which were induced by exogenous AI-2 and were subsequently inhibited when a low amount of furanones was added to the cultures. AI-2 signaling inhibition is poorly known in gram-positive bacteria, since no specific signaling pathway have yet been described, even if staphylococcal biofilm growth was significantly inhibited on materials used in medical applications which contained a modified surface functionalized with furanones (Lee *et al.*, 2005; Singh *et al.*, 2007).

3.3.1.4 Plant-derived Virulence Modulators

Since ancient times, plants have proved to be effective in their healing and antimicrobial properties. Although the specific action of plant-derived compounds on the development of bacteria at a molecular level is largely unknown, studies have reported that they may attenuate bacteria virulence, adherence and biofilm formation and also modulate QS communication (Anghel *et al.*, 2012b). Plant extracts and essential oils obtained from *Rosmarinus officinalis, Mentha piperita, Foeniculum vulgare, Salvia officinalis, Eugenia caryophyllata, Citrus maxima, Picea abies, Anethum graveolens, Abies alba, Pseudotsuga menziesii, Larix decidua* and *Pinus nigra* have proved to be active on many fungal, gram-positive and gram-negative bacterial strains. Because of their potential to exert antimicrobial activity, in recent years many pharmaceutical companies have focused their activity on identifying and developing the best formulations for a desired purpose, thus opening perspectives on a new era of natural and ecologic therapies(Holban *et al.*, 2014a). Since 2000, more than 300 plant-derived compounds with great antimicrobial activity have been described. The most investigated groups of compounds with antimicrobial activity are alkaloids, acetylenes, coumarins, flavonoids, iridoids, lignans, macrolides, polypeptides, quinones, steroidal saponins, terpenoids, xanthones, miscellaneous compounds and other phenolics (Ghani *et al.*, 2008; Saleem *et al.*, 2010). These compounds alone or in a mixture (i.e as they are

found in the composition of essential oils) have shown different activities against the virulence, attachment, biofilm formation and viability of most pathogenic bacteria.

Essential oils, also known as volatile oils, are concentrated hydrophobic liquids containing volatile aromatic compounds extracted from plants. Studies have revealed that rose, geranium, lavender, peppermint, cinnamon, clove and rosemary oils are very potent QS inhibitors mediating QS dependent phenotypes in gram-negative species. Clove, cinnamon, lavender and peppermint oils revealed a promising anti-QS activity in *Chromobacterium violaceum*, significantly inhibiting pigment production. Essential oils from clove have proved to interfere also with *P. aeruginosa* QS, reducing social virulence phenotype of swarming motility (Khan *et al.*, 2009).

Even though the molecular mechanisms by which essential oils and their major volatile compounds interfere with bacteria QS are mostly unknown, our recent studies demonstrate that *Rosmarinus officinalis, Mentha piperita, Salvia officinalis, Eugenia caryophyllata* and *Citrus maxima* essential oils directly modulate the expression of genes involved in QS circuits in S. *aureus* and *P. aeruginosa*. Saviuc and collaborators demonstrate that *E. caryophyllata* essential oil down-regulated the expression of *rhlI, rhlR, lasI* and *lasR* genes, which are key AHL- mediated QS signaling genes in *P. aeruginosa*. Also, *S. officinalis, R. officinalis* and *E. caryophyllata*, essential oils, and limonene and eugenol volatile compounds have proved to significantly repress *agrI* gene in *S. aureus* demonstrating molecular interference of essential oils with bacteria QS circuits *in vitro*. Furthermore, the results revealed that *S. aureus* and *P. aeruginosa* virulence is significantly repressed when bacteria is grown in medium containing essential oils, since production of soluble virulence factors, such as exoenzymes and toxins are inhibited (Saviuc *et al.*, 2013).

In vivo studies demonstrate that plants may produce many potential bacteria QS and virulence inhibitors during infection. It have been shown that in growing onion bulbs infected with *P. aeruginosa*, several compounds like pantolactone, 4,5-dihydro-4,5-dimethylfuran-2(3H)-one, myristic acid, and linoleic acid are produced. It was suggested that these compounds may be produced by plants and act as an efficient defense system. Pantolactone and myristic acid have have shown potent QS modulator activity in *P. aeruginosa* by significantly inhibiting some of the main virulence determinants involved in the pathogenicity of this species, such as pyocyanin production, protease, lipase and polygalacturonase activity, without an impact on bacterial growth (Abd-Alla & Bashandy, 2012).

Various species of the vegetal taxon *Dalbergia* are traditionally used for sundry ailments and some of them have recently been shown to quench the virulence of gram-positive and gram-negative bacteria. N-hexane extracts of leaves, roots and barks of endemic malagasy *Dalbergia* species have shown an enhanced capacity to antagonize QS mechanisms in *P. aeruginosa* by reducing the expression of the QS-regulated genes *lasB* and *rhlA* (Rasamiravaka *et al.*, 2013). However, studies have demonstrated that just the extract of *D. trichocarpa* bark showed a significant reduction of QS-gene expression without affecting *aceA* gene encoding a QS-independent isocitrate lyase. Furthermore,

D. trichocarpa bark treatment inhibited biofilm formation and even to disrupt mature biofilms. Preliminary structural characterization of these potent biofilm disrupters suggests that they belong to the phytosterols, a class of vegetal hormones involved in cell-to-cell signaling. Further characterization of *D. trichocarpa* bark impact on QS revealed that the QS systems *las* and *rhl* were inhibited and the associated phenotypes significantly modulated. Swarming and twitching motilities, biofilm formation and the production of pyocyanin, elastase and proteases were hampered in the presence of the *D. trichocarpa* bark extract. The strong inhibition of motility and biofilm formation suggests that this extract contains agents able to disrupt the architecture of biofilms, which is an important observation in the context of the design of new drugs targeting biofilm-encapsulated pathogens that are very resistant to standard treatment (Rasamiravaka *et al.*, 2013).

In a recent study, Anghel and collaborators demonstrated that several purified compounds derived from plants can be successfully used to modulate microbial biofilms formation on the surface of wound dressings and prosthetic device associated infections. Their studies demonstrated that *Anethum graveolens* and *Salvia officinalis* essential oils have great antifungal abilities, combating *C. albicans* infections. When immersing wound dressings in these essential oils, the resulting medical structures have increased resistance to *C. albicans* colonization, reducing both the adherence and biofilm formation of this pathogen (Anghel *et al.*, 2013c). *Mentha piperita* essential oil has also proved great anti-adherence properties against Staphylococcal colonization on the surface of modified prosthetic devices. A recent study demonstrates that antibacterial effect of *M. piperita* extract is also extended on biofilms formation by reducing *S. aureus* biofilm initialization and maturation for up to 72 h (Anghel & Grumezescu, 2013a).

Ethanol extracts of *Amburana cearensis and Anadenantheramacrocarpa* had proved a great anti-infectious effect, enhancing the activity of several antibiotics against multiresistant *S. aureus* and *E. coli* strains isolated from different human infections (Figueredo *et al.*, 2013). In this study the authors concluded that these ethanolic extracts can be used as an alternative source of natural products with antibacterial action because of the presence of several antimicrobial fractions, which can be responsible for the observed modulatory effects, indicating the possibility of using natural products combined with aminoglycosides to increase the antimicrobial potential of these drugs against multiresistant microorganisms.

3.3.1.5 Animal-derived Virulence Modulators

Animal hosts have also adapted to bacterial pathogens for a better survival. Since QS inhibitors have proved to exist in plants naturally, and have been shown to attenuate bacteria virulence and infectivity; researchers have next explored animal - derived signaling compounds with the aim of finding molecules to protect human and

animal hosts against infections by modulating pathogen virulence and cell-to-cell communication.

Immediately after entering the host cells, bacteria QS molecules are inactivated or cleaved by mammalian enzymes, most of which are currently unknown. QS inactivation seems to be molecule specific. A study performed on human epithelial airway tissues revealing that after treatment with *P. aeruginosa* AHLs, 3OC12-HSL is cleaved shortly after addition, but not C4-HSL (Chun *et al.*, 2004). Human paraoxonases are highly conserved antioxidant enzymes, which have also been proved the ability to hydrolyze lactones.

Despite their antioxidative properties, the presence of three highly conserved lactonases in animals in the absence of any significant endogenous lactones was considered intriguing and researchers aimed to identify natural substrates for these enzymes. It has been proved that paraoxonases can interfere with bacteria QS, degrading *P. aeruginosa* that produces AHLs and through blocking this bacterium communication also reduce the extent of infection (Ozer *et al.*, 2005).

In recent years, some mammalian cell-to-cell signaling molecules have proved to interfere with bacteria virulence and QS communication. It has been demonstrated that the pathogens are able to recognize and respond to various host signaling molecules, such as the opioid dynorphin, brain natriuretic peptide (BNP) hormones (Veron *et al.*, 2008), IFNγ (Wu *et al.*, 2005) and also catecholamines (Hegde *et al.*, 2009; Karavolos *et al.*, 2011).

Recent data demonstrate that *P. aeruginosa* can intercept opioid compounds released during host stress and integrate them into core elements of QS circuitry leading to enhanced virulence. The natural k-opioid peptide hormone, dynorphin, and some of its synthetic non-peptide analogues (e.g. U-50,488) proved to increase the virulence of *P. aeruginosa*, acting as a QS quinolonic derivative. Dynorphin and U-50,488 signaling pathway is related with the control of GacA and MvfR proteins, which regulate the expression of *pqsABCDE* and *phzA1-G1* operons involved in the synthesis of pyocyanin and PA-1 adhesin. These virulence factors may damage the host epithelium on site and promote neutrophils activation. Furthermore, the high amounts of quinolone AIs produced after the activation of *pqsABCDE* operon, proved to "attack" probiotics like *Lactobacillus* sp., by interrupting their normal communication (Zaborina *et al.*, 2007). This strategy aims to eliminate the competitor commensal bacteria and to facilitate the colonization of intestinal tract with pyocianic bacillus. Furthermore, *in vivo* significance of kappa-opioid signaling of *P. aeruginosa* was demonstrated in mice by showing that dynorphin can be released from the intestinal mucosa following ischemia/reperfusion injury, activates quinolone signaling in *P. aeruginosa*, and enhances its virulence against probiotic species of *Lactobacillus* and nematode *Caenorhabditis elegans* (Zaborina *et al.*, 2007).

Brain natriuretic peptide (BNP) and C natriuretic peptide (CNP), known as guanylyl cyclase activators and adenylyl cyclase inhibitors in mammals, proved to have a bactericidal activity on gram-positive bacteria (Krause *et al.*, 2001). On the other

hand, recent studies demonstrate that BNP and CNP may be involved in the control of *P. aeruginosa* and *P. fluorescens* virulence, without affecting the survival rate of these gram-negative bacteria (Veron *et al.*, 2008). The results demonstrated that natriuretic peptides significantly reduce the ability of *P. fluorescens* to promote the apoptosis of human glial cells, but in the same time increase the capacity of both *P. aeruginosa* and *P. fluorescens* to promote necrotic changes within the host glial cells. The explanation of this effect is related to the fact that the natriuretic peptides control the synthesis and secretion of some cytotoxic enzymes, like phospholipase C, which induce host cells necrosis (Boon *et al.*, 2008). BNP and CNP activate cyclic Adenosine Mono-Phosphate (cAMP) and cyclic Guanylate Mono-Phosphate (cGMP) dependent bacterial signaling pathways, acting similarly in both prokaryotic and eukaryotic cells. In *P. fluorescens* and *P. aeruginosa* the target of natriuretic peptides is the synthesis of the virulence modulator – Vfr protein, which does not discriminate between cAMP and cGMP and is activated by both (Beatson *et al.*, 2002). The activation of Vfr protein leads to an enhanced production of virulence factors and quorum sensing signaling molecules (QSSMs), which in turn, modulate the behavior of the bacterial population. *S. typhimurium, Shigella flexneri*, *Erwinia chrysantemi* and *E. coli* are also able to recognize and respond to hosts peptide hormones, however, the molecular recognition pathways are largely unknown (Peschel, 2002). Recent studies demonstrate that *Burkholderia pseudomallei* may recognize and use insulin to modulate the physiology in diabetes mellitus patients, although further experiments revealed that insulin clearly inhibited the growth of this bacterium. Despite these results, the existence of a specific receptor for insulin in *B. pseudomallei* remains controversial (Woods *et al.*, 1993). The gastric pathogen *Helicobacter pylori* proved to be responsive to high amounts of gastrin and somatostatin, usually encountered in the stomachs of infected individuals. Human gastrin stimulated the growth of *H. pylori* in a specific and dose-dependent manner. Gastrin is recognized by a particular receptor, the structure of which is still unknown, has only been found in *H. pylori* (Chowers *et al.*, 2002). On the other hand, somatostatin significantly inhibits the growth of *H. pylori* by increasing the intrabacterial concentration of cGMP and cAMP. Even though the molecular structure of the recognizing receptor has not yet been found, the fact that somatostatin neither binds to other gram-negative bacteria nor affects their proliferation, indicates that the expression of this bacterial sensor is restricted to specific bacterial species and suggests that a specific cross-talk occurs between eukaryotic cells and *H. pylori* (Hofland & Lamberts, 2003).

Studies investigating the role on neuroendocrine stress hormones on bacteria species suggested that catecholamine hormones may act as a bacterial QS surrogate molecules by developing similar effects with peptide bacteria autoinducers, generically called hormone-like molecules (Hofland & Lamberts, 2003; Holban & Lazar, 2011). It was demonstrated that catecholamine hormones such as adrenaline, noradrenaline, dopamine, inotropic isoprenaline, dobutamine and their metabolites (i.e. dihidroximanedelic and dihidroxifenilglicol acid) and plant extracts containing

catechol-type compounds (e.g. tannic acid, chlorogenic, caffeic, catechins) have the ability to stimulate bacterial growth (Bearson & Dowd, 2010; Holban *et al.*, 2013). Based on the observation that intramuscular injection of adrenaline in humans can lead to rapid gas gangrene produced by *Clostridium welchii* at the site of injection, Evans and co-workers have shown that the number of microorganisms necessary to produce infections is substantially reduced when they are administered with adrenaline under experimental conditions. In addition to reducing the minimum infectious dose, researchers have found that adrenaline enhances the clinical manifestations of the infection (Evans *et al.*, 1948).

Studies have shown that in minimal cell culture medium containing serum, catecholamine neurohormones can stimulate the growth and modulated virulence of different pathogenic and opportunistic bacteria. So far, two mechanisms have been proposed to explain the catecholamine stimulation of bacterial growth. The first mechanism seems to be dependent on the host molecules and suggests that catecholamine hormones stimulate bacterial growth by facilitating iron uptake *via* their binding to the host serum iron binding proteins, such as lactoferrin and transferring (Sandrini *et al.*, 2010). The second mechanism appears to be independent of the host molecules and is based on the *in vitro* production of thermostable bacterial growth autoinducers in the presence of catecholamines (Freestone *et al.*, 1999).

Besides having an impact on the bacterial growth, catecholamine stress hormones also play an important role in modulating bacterial virulence and physiology in certain experimental conditions. Studies revealed that noradrenaline (norepinephrine, NA) can upregulate the expression of genes involved in the production of fimbriae and toxins in pathogenic *E. coli* strains (Burton *et al.*, 2002; Lyte *et al.*, 1996). The induction of *E. coli* growth in the presence of catecholamines is dependent on the presence of an intact enterobactin uptake system. This suggests that adrenaline (epinephrine, A) and NA are involved in the induction of enterobactin expression, the mechanism responsible for *E. coli* growth stimulation in tested strains. However, the role of NA in bacterial pathogenesis appears to be more complex as the signaling mechanisms and iron acquisition overlap. For example, the siderophore pyoverdin from *P. aeruginosa* can fulfill the role of signaling molecule, being involved as well in the iron uptake (Burton *et al.*, 2002).

The role of A and NA signaling molecules in the bacterial pathogenesis is supported by studies showing that these hormones are involved in the synthesis of flagella and type III secretion system (T3SS) in enterohaemorrhagic *E. coli* (EHEC) O157: H7 (Sperandio *et al.*, 2003). It seems that EHEC has the ability to detect and use host derived A and NA, and aromatic bacterial QSSMs as autoinducer 3 (AI-3), in order to enable the virulence mechanisms, suggesting that that these signals are interchangeable. The same research group showed that A and NA may act as surrogate QS molecules and may develop similar effects of bacterial molecules acting as autoinducer peptides, commonly referred to as hormone-like molecules. The recognition of these three signals: A, NA and AI-3 is essential for the *in vivo* virulence expression of EHEC.

In addition, authors indicate QseC as a common receptor for catecholamine, especially NA and the newly described bacterial autoinducer AI-3. They also propose that the two component system QseEF as being involved in adrenergic recognition in *E. coli*. Furthermore, AI3, NA and A function as antagonists and the response of *E. coli* to these signaling molecules can be blocked by using adrenergic antagonists (Sperandio *et al.*, 2003).

Karavolos and collaborators observed that exposure of *S. typhi* to neuroendocrine hormones resulted in increased haemolytic activity. A proteomics-based dissection of the haemolytic phenotype identified a significant reduction in the levels of outer membrane protein A (OmpA) after exposure to physiological concentrations of adrenaline or noradrenaline. This observation is attributed to increased levels of the small RNA (sRNA) chaperone protein Hfq and the sRNA micA repressing ompA expression. The haemolytic response was specific to membrane vesicles, and was not observed in *S. typhi* strain lacking the sRNA, mica (Karavolos *et al.*, 2011). The authors also revealed that these effects could be reversed by the addition of the β-adrenergic blocker propranolol. Another remarkable finding is that the neuroendocrine hormone-mediated haemolysis required the CpxAR two-component signal-transduction system and was independent of the *E. coli* O157:H7 bacteria adrenergic receptor orthologue QseBC, the only bacteria adrenergic signaling pathway reported so far (Karavolos *et al.*, 2008).

Another bacterial pathogen that seems to sense and respond to adrenergic hormones by QS – dependent modulation of virulence, *is P. aeruginosa*. Li and collaborators demonstrated that NA reduce the virulence of *P. aeruginosa* PAO1 strain *in vitro* through the suppression of genes involved in the production of exotoxin A and siderophores (Li *et al.*, 2009b). These results are in contrast to previous studies performed on other bacterial species which revealed that catecholoamines stimulates the expression of virulence genes (Karavolos *et al.*, 2011; Sperandio *et al.*, 2003). The stress hormone norepinephrine increases growth, virulence factors production, ability to invade the HCT-8 epithelial cells and swimming motility of *P. aeruginosa* PA14 strain in a concentration dependent manner. Transcriptome analysis of *P. aeruginosa* exposed to 500 μM, but not 50 μM, norepinephrine for 7 h showed that genes involved in the regulation of the virulence determinants pyocyanin, elastase, and the signaling QS molecule PQS were upregulated (Hegde *et al.*, 2009). In addition, the production of rhamnolipids, which represents a key factor in *P. aeruginosa* infections, was not significantly altered in suspension cultures upon exposure to 500 μM norepinephrine, but decreased on semisolid surfaces. Swarming motility, a phenotype that is directly influenced by rhamnolipids, was also decreased upon 500 μM norepinephrine exposure. Hedge and co-workers revealed that the increase in the transcriptional activation of *lasR* but not that of *rhlR* and the increase in the levels of PQS suggest that the effects of norepinephrine are mediated primarily through the *las* quorum-sensing pathway (Hegde *et al.*, 2009). In contrast, another research group demonstrated that catecholamines are able to inhibit *P. aeruginosa* virulence by repressing the expression of *toxA* and the siderophore genes. It has been revealed that norepinephrine enhances the growth of bacteria

by supplying iron from serum iron binding proteins like transferrin. This provision of iron seems to repress the expression of exotoxin A gene, *toxA*, the pyoverdine genes *pvdD* and *pvdE*, and their regulators, *pvdS*, *regA*, and *pchR*, suggesting that norepinephrine accomplishes this repression through PvdS and PchR regulatory proteins (Li *et al.*, 2009a).

Even though there is a great interest, controlling bacterial virulence by natural QS modulators is still a new and poorly explored field, with great promise for biomedical applications.

3.3.2 Synthetic Virulence and QS Signaling Modulators

Since technological progress brings novel insights into discovering the intimate molecular support of bacterial QS signaling molecules, researchers aim to develop targeted synthetic QS modulators. In *S. aureus,* QS signaling is controlled by *agr* system, which controls the production of Agr peptides that have been found to contain an unusual thiol ester-linked cyclic structure. Mayville *et al.* demonstrated that synthetic Agr thiolactone-containing autoinducing peptides interfere with *S. aureus* virulence both *in vitro* and *in vivo* (Mayville *et al.*, 1999).

Recent work on modulating AHL signaling in gram-negative bacteria lead to the identification of novel small molecule inhibitors binding to N-acyl-homoserine lactone synthase TofI in *Burkholderia glumae*. In this bacterium, the main AI synthesized by TofI is C8-HSL, which seems to control virulence, motility, and protein secretion. Chung and coworkers characterized two previously unknown QS inhibitors identified in a focused library of acyl-HSL analogues. Their functional and X-ray crystal structure analyses showed that the first inhibitor, J8-C8, binds to TofI, occupying the binding site for the acyl chain of the TofI cognate substrate, acylated acyl-carrier protein. Closer inspection of the mode of J8-C8 binding to TofI provides a likely molecular basis for the various substrate specificities of acyl-HSL synthases. The second inhibitor, E9C-3oxoC6, competitively inhibits C8-HSL binding to TofR, the cognate receptor of C8-HSL (Chung *et al.*, 2011). Singh *et al.* (2005) proposed several femtomolar transition state analogue inhibitors of 5'-methylthioadenosine/Sadenosylhomocysteine nucleosidase (MTAN) from *E. coli*. 5'-Methylthio-Immucillin-A (MT-ImmA) derivatives have proved to be efficient inhibitors for MTAN. Substitution of the methylthio group with a p-Cl-phenylthio group gives a more powerful inhibitor since it provides a better dissociation constant $K_{(i)}$. Among tested synthetic inhibitors, the most powerful inhibitor was 5'-p-Cl-phenylthio-DADMe-Immucillin-A (pClPhT-DADMe-ImmA) with a $K_{(i)}$ value of 47 fM (47×10^{-15} M). These are among the most powerful non-covalent inhibitors reported for any enzyme, binding 9-91 million times tighter than the MTA and SAH substrates in *E. coli*. The inhibitory potential of DADMe-Immucillins has proved to support a fully dissociated transition state structure for *S. pneumoniae* MTAN. Therefore, powerful inhibitors of MTAN are candidates to disrupt key bacterial pathways including

methylation, polyamine synthesis, methionine salvage, and quorum sensing (Singh *et al.*, 2005).

Other studies reported that in *P. aeruginosa*, a structurally unrelated triphenyl stabile mimic seems to interact specifically with AHL QS circuit. The triphenyl mimic seems to interact specifically with LasR but not with QscR. *In silico* analysis suggests that the mimic fits into the 3OC12-HSL -binding site of LasR and makes key contacts with LasR. Same authors also suggest that the triphenyl mimic can be used as an excellent scaffold for developing quorum-sensing inhibitors, and its stability and potency make it ideal for biotechnology uses such as heterologous gene expression (Muh *et al.*, 2006).

Even though is a recent described molecule, AI-3 control of virulence in *E. coli* has encouraged investigators to develop small molecules that could inhibit this signaling system. Rasko *et al.* (2008) have recently described the identification of one such molecule, through a high-throughput screening of a large library of compounds. The authors identified N-phenyl-4-phenylaminothioxomethylamino-benzenesulfonamide (LED209) as an inhibitor of QseC and bacterial virulence, both *in vitro* and *in vivo*. LED209 inhibits QseC autophosphorylation, virulence factor production and Attaching Effacing lesion formation by EHEC. Additionally, LED209 can inhibit virulence factor production and host colonization by *S. typhimurium* (Antunes *et al.*, 2010; Rasko *et al.*, 2008).

4 Increasing the Efficiency of Antimicrobial Compounds Using Nanotechnology

Even if many natural and synthetic compounds have proved their great efficiency in antimicrobial approaches or in modulating microbial virulence and communication, their medical use is restricted by certain physicochemical characteristics. Despite their proved antimicrobial effect, their specific signaling pathways at molecular level remain largely unknown. The lack of knowledge regarding the specific effect of natural compounds on bacterial cells is mainly because of some biochemical traits they exhibit, such as low stability, high volatility, great diffusibility and instability. These factors lead to the necessity of developing vectorizing agents or molecular shuttles for improving their efficiency, investigating their specific effect, and develop new and optimized investigation methods adapted to their specific properties (Grumezescu *et al.*, 2013a; Voicu *et al.*, 2013). Obtaining appropriate vectors for the delivery and controlled release of volatile compounds and essential oils may be a difficult task since bioactive compounds have different physico-chemical properties. Recent studies are focusing on stabilizing and reducing volatility of essential oils and plant-derived active compounds, which are usually volatile and instable.

Researchers are working on enhancing essential oils stability by developing nanostructured vectoring systems and by proposing improved synthesis, functionalization, characterization and biological effect assessment methods (Grumezescu *et al.*, 2012b; 2012c; Saviuc *et al.*, 2011; 2012). Apart of stabilizing active volatile compounds, nanohybrid phytoactive systems can also be used for controlled release and specific targeting of both natural and typical synthetic antimicrobial agents, such as antibiotics (Anghel *et al.*, 2012a).

4.1 Nanostructures Used in Anti-infectious Therapy

Nanotechnology is an interdisciplinary field involving extensive knowledge in various fields of science (like physics, chemistry, mathematics, biotechnology and materials science) and it is considered the technology of the future that allows observing, measuring, understanding and handling different structures and processes at a nanometric scale (Ramsden, 2011; Zeineldin, 2014; Zhao & Qian, 2011). Due to their small size (1-100 nm) ease of modification of structural and functional characteristics, nanoparticles play an important role in the scientific world as macrostructure connection between atomic and molecular structures (Boulaiz *et al.*, 2011). The material properties are dependent on the nano-level size, modifying the characteristics of the material as the size approaches the nanometer range and as the percentage of surface atoms becomes significant. The materials designed to develop nanoscaled structural properties are morphologically and functionally unique and exhibit a high value of surface/

volume ratio (Wu *et al.*, 2008). In order to obtain nanoscale sized particles, nanotechnologies have enabled the realization of two main directions, which allows for the synthesis of nanoparticles in liquid, solid or vapor phase: i) bottom-up approach (for the synthesis of nanoparticles from macrodimensioned materials) and ii) top-down approach (for the nanodimensioned particle synthesis from molecular or atomic structures) (Mendes, 2013). Nanoscaled particles may be developed with customized structures and properties depending on their size and shape and the versatility of use in various fields can be influenced by the synthesis method (Mitragotri & Lahann, 2009).

4.1.1 Zinc Oxide Nanoparticles

Zinc oxide (ZnO) has been used in various applications for thousands of years and it can be reasonably considered a mature engineering material with an annual production approaching 1.5 million tones. ZnO was used from ancient times as a constituent of medicinal ointments for treating inflammation and *S. aureus* infections (Biswas, 1986; Habashi, 2002).

Applications of the ZnO nanopowders are numerous, most applications exploiting the reactivity of the oxide as a precursor to other zinc-based compounds. For materials science applications, zinc oxide has a high refractive index, high thermal conductivity, binding properties, antibacterial and UV protection. Therefore, it is added to materials and products, including plastics, ceramics, glass, cement, tires, lubricants, paints, dyes, ointments, adhesives, sealants, pigments, batteries, ferrites and fire resistant materials (Klingshirn, 2007).

ZnO as a mixture with 0.5% iron oxide is called *calamine* lotion. When mixed with the plant derived compound eugenol, which is a ligand, the complex formed from zinc oxide-eugenol is used as a tonic and a dental prosthetic component.

ZnO is widely used for the treatment of various dermatological disorders in products such as baby powders, creams for treating rashes, calamine lotion, anti-dandruff shampoos and antiseptic ointments. Zinc oxide can be used in lotions for protecting against sunburn caused by UV radiation. It is the broadest UVA and UVB spectra reflector that is approved for use in sunscreen products by the FDA and is completely photostable.

ZnO nanoparticles can enhance the antibacterial activity of certain antibiotics, such as ciprofloxacin. It has been shown that nano ZnO with average particle size between 20 and 45 nm may enhance the antibacterial activity of ciprofloxacin against *S. aureus* and *E. coli in vitro*. The enhancing effect of this nanomaterial is dependent on the concentration and time. Antimicrobial effects of ZnO have been monitored against gram-positive, gram-negative and fungal strains, and each time result was the same: very powerful microbicide (Chauhan *et al.*, 2014).

4.1.2 Silver Nanoparticles

Due to the specific biocidal activity, silver has been accorded a wide range of practical applications in conducting daily activities over time. The first historical records of certainty that certifies the use of silver for medical purposes are attributed to Gabor, around 702-705 BC (he mentions the curative potential of silver nitrate in treating wounds) and later by Avicenna, in the 980s BC (which mentions the use of silver filings in order to purify infected blood and for the prevention and treatment of respiratory diseases).

Studies conducted at the end of the nineteenth century by the Swiss botanist Karl Wilhelm Von home Nägeli proved the antimicrobial efficacy of silver against more than 650 species of microorganisms, which lead to its excessive use in the treatment of many infectious diseases in the first half of the twentieth century and early reporting of adverse effects on the human body silver (Alexander, 2009; Nedelcu *et al.*, 2014).

In order to use the silver particles in the current medical applications, nanotechnology enables the synthesis of high purity particles characterized by physico-chemical properties, with dimensions ranging 2-100 nm and various shapes (spherical, triangular, hexagonal, cubic, tetragonal, ribbon, wire, rod forms) that are controlled by changing the process parameters during synthesis (Gunasekaran *et al.*, 2011). Recent advances in technology offers the possibility of the synthesis of silver nanoparticles with characteristic properties, original dimensionally developed at this level are due to the high specific surface area, in relation to the reduced contribution of the volume, the electrical conductivity, chemical stability, catalytic activity, antibacterial, antifungal, antiviral and anti-inflammatory activities (Tiwari *et al.*, 2011). These characteristics attributed to the silver nanoparticles have been found to be optimal for a wide range of applications in biophysics, biochemistry, electrochemistry, electrical engineering, optics, electronics, molecular biology, pharmacology and medicine (Sironmani & Kiruba, 2011).

Promising results from studying interactions between silver nanoparticles and living organisms, experimentally obtained toxicity data and the cellular distribution of these particles shows the versatility of silver nanoparticles to be used in many biomedical applications, such as detection and destruction of pathogenic microorganisms, biological synthesis of fluorescent ligands for specific markers involved in diagnosis, pharmacokinetic and pharmacodynamic studies, medical imaging, drug therapy, gene therapy, production of biosensors, localization and destruction of neoplastic cells by hyperthermia, sterilization of implants and antimicrobial treatment of household materials (Chichova *et al.*, 2014; de Faria *et al.*, 2014; Kaler *et al.*, 2014; Kheiralla *et al.*, 2014; Sironmani & Kiruba, 2011; Zhao *et al.*, 2014).

Although the specific mechanism of antimicrobial silver nanoparticles on the pathogens is not yet fully understood, a number of specialized studies have been performed to describe the interactions between silver nano particles and microbial agents along with phenomena that occur at the nano level have outlined a three stage

hypothesis for the biocidal effect of silver nanoparticles (Chowdhury *et al.*, 2014). Surface reactivity of silver nanoparticles – due to their small size – favors the formation of strong links between the particles and the microbial membrane components containing sulfur or phosphorus; from the anchoring surface of the silver particles, their size in the nanometer range favors the displacement of essential cell survival ions (K^+, Ca^{2+}, Zn^{2+}), thus changing the microbial membrane permeability (Quang Huy *et al.*, 2013). Interaction of silver nanoparticles with the membrane of micro-organisms favors the release of metal ions, which affect the activity of structures containing phosphorus, nitrogen or sulfur – such as the microbial proteins, enzymes, and nucleic acids (Sekhon, 2014). In addition to the two steps mentioned above, the microorganisms are subject to enhanced oxidative stress upon interaction with silver nanoparticles. The mechanical damage and loss of structural integrity of the pathogen is favored by the formation of intracellular deposits of silver nanoparticles and release of metal Ag^+ ions. The presence of the silver ions at this level blocks the transport of electrons involved in the synthesis of ATP (adenosine triphosphate) and promotes the generation of reactive oxygen species (Rai *et al.*, 2009). The use of silver nanoparticles as potential antimicrobial agents in biomedical applications is derived from the current biocidal action exhibited by these agents on many types of pathogenic bacteria (*E. coli, V. cholerae, S. typhi, P. aeruginosa, E. faecalis, S. aureus* etc.), fungi (*C. albicans, Trichophyton rubrum, Trichophyton mentagrophyte, Aspergillus* sp., *Penicillium* sp.) and viruses (human immunodeficiency virus HIV-1, hepatitis B virus, influenza A virus) (Kim *et al.*, 2007; Quang Huy *et al.*, 2013). The efficient recovery of biocidal silver nanoparticles allows its use in the medical field: disinfection and coverage of medical devices, incorporating particles into dermal patches development of dental and orthopedic cements and the formulation of substances for topical administration (Kim *et al.*, 2007; Prabhu & Poulose, 2012; Rai *et al.*, 2009).

4.1.3 Magnetite Nanoparticles

Magnetic nanoparticles, such as Fe_3O_4, are intensively studied due to their small dimensions and exceptional properties, which make them suitable for biomedical applications. Magnetite is a well-known material studied for decades, but in the past few years scientists have increasingly started using magnetite nanoparticles for medical applications. Different types of applications, such as MRI contrast enhancement, magnetic hypertermia, drug-delivery or targeting, antimicrobial therapy, etc., have been developed. Magnetic nanostructures designed for biomedical applications must be non-toxic, biocompatible and chemically stable (Grumezescu *et al.*, 2013c; Grumezescu *et al.*, 2012ad, 2012b). Chemical stability of magnetic nanostructures is usually achieved by coatings of the surface with different compounds such as small organic molecules (e.g. citrate, surfactants, and fatty acids), large organic molecules (e.g. polymers, proteins, polyelectrolytes, copolymers – polyethylene glycol, pluronic,

dextran, chitosan) or inorganic elements (e.g. silica, alumina). This process in called *functionalization* and is important to achieve specific properties after the nanoparticles synthesis. Another important factor which should be considered in the design of magnetite nanoparticles intended for biomedical applications is to prevent aggregation of particles and to stabilize the dispersion by steric or electrostatic repulsion (Figure 4.1) (Illés *et al.*, 2014).

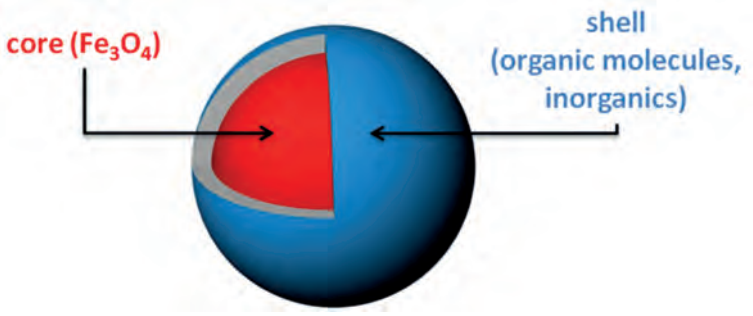

Figure 4.1: Functionalized magnetite nanostructures (Illés *et al.*, 2014).

In the last few years, two novel biomedical applications were developed using magnetic nanostructures: i) enhancements of antimicrobial activity of different molecules by the presence of functionalized magnetic nanoparticles; ii) modulation of microbial colonization and biofilm development by the presence of functionalized magnetic nanoparticles.

4.1.3.1 Synthesis of Fe_3O_4 Nanoparticles

For most applications, choosing the best synthesis route represent an important step, conditioned by the dimension of the particles, shape, size distribution, chemistry of the surface, etc. During the time numerous synthetic routes have been developed, such as co-precipitation, hydrothermal, solvothermal or sonochemical methods. From the available synthetic procedures, the preferred method of synthesis in a research setting is the co-precipitation approach, offering a safe synthesis method with very good reproducibility and offering a good control on the particle dimensions. This route was developed by Massart in 1981, by mixing Fe(II) with Fe(III) in the presence of HO^- (Massart, 1981). Co-precipitation process is carried out in two steps: i) the nucleation process; and ii) the growth of nuclei. pH has an influence on the formation of magnetic nanoparticles with an optimum pH ranging between 9-14 (Figure 4.2). The co-precipitation must be conducted in a non-oxidant media, due to the presence of the atmospheric oxygen that transform the magnetite in *maghemite*, *via* oxidation (Lodhia *et al.*, 2010). The principal advantage of co-precipitation route is that it can be used to prepare a large amount of magnetite nanostructures, but in order to obtain a good

distribution of the nanostructures dimensions, different parameters must be adjusted; such as: i) molar ratio of Fe(II)/Fe(III); ii) value of pH; iii) concentration of ferric and ferrous salts; and iv) temperature of reaction. For a rigorous control of the particle size, different surfactant or fatty acids such as: sodium oleate, sodium dodecyl sulfate and mercaptoethanol may be used (Meng *et al.*, 2013).

Figure 4.2: Synthesis of Fe_3O_4 through co-precipitation route.

4.1.3.2 Functionalized Magnetite Nanoparticles

Nanoparticles without any surface modification present a series of disadvantages namely: i) aggregation in water; ii) chemical instability when exposed to air; iii) reduced biodegradability in physiological environment; iv) non-specific interactions with plasma proteins; v) agglomeration of nanoparticles *in vivo*; and vi) fast elimination of nanoparticles *via* the immune system (Muthiah *et al.*, 2013).

There are two methods used to develop covers for magnetite nanoparticles: i) addition of the ligands, through physical adsorption on the surface of magnetite nanoparticles due to electrostatic forces, hydrophobic interactions or hydrogen bonding formation; and ii) ligands exchange, which are explained by the fact that some functional groups from the initial surface of nanoparticle are replaced with functional groups such as -OH, -NH_2, -COOH, -SH (Muthiah *et al.*, 2013).

Opsonization, due to the hydrophobic character of nanoparticles, may be prevented by decorating the surface of particles with hydrophilic molecules such as polyethylene glycol (PEG), dextran, cellulose, polyvinyl alcohol (PVA), chitosan, alginates, polyvinylpyrrolidone (PVP), etc.

PEG is the most used polymers in the fabrication of micro and nanostructured systems based on magnetite nanoparticles, presenting the following advantages: i) high solubility in aqueous media; ii) minimize of opsonization phenomena; iii) acts as a spacer for different molecules and targeting ligands; this helps to minimize the non-specific interactions, favoring intratumoral accumulation through the high retention and permeability phenomena (Muthiah *et al.*, 2013).

Saviuc *et al.*, reported a novel hybrid nanomaterial based on PEG, magnetite and *Citrus maxima* essential oil. This type of nanohybrid presents an impressive

antimicrobial activity against *S. aureus*, *E. coli, E. faecalis* and *K. pneumoniae* with a minimum inhibitory concentration (MIC) up to 0.072 mg/mL. PEG functionalized magnetite nanoparticles showed a reduced microbial adherence ability to the cellular substrata with a diffuse aggregative adherence pattern to the HeLa cells (Saviuc *et al.*, 2011).

Dextran, is a bacterial-derived polysaccharide that has received an increased attention due to the variety of supported applications such as drug targeting and delivery, low tissue toxicity and high enzymatic degradability at the desired sites (Kamoun *et al.*, 2014).

Grumezescu *et al.* (2012a) recently reported the preparation of dextran-magnetite-silica microspheres as a drug carrier for the delivery of different antimicrobial substances. This type of composite prepared by co-precipitation represents a novel approach to antimicrobial therapy. The encapsulation of vancomycin, clindamycin, azithromycin, oxacyllin, trimethoprim/sulfamethoxazole, rifampicin, ofloxacin, tetracycline, penicillin, ciprofloxacin, gentamycin, piperacillin/tazobactam, cefepime, aztreonam, ceftazidim and piperacillin have been reported successfully. In the case of *S. aureus,* excepting vancomycin, aztreonam and ofloxacin, whose antimicrobial activity was not influenced, the prepared microsystem exhibited a potentiating effect on the antimicrobial activity of all other anti-staphylococcal agents, as revealed by an increase of the growth inhibition zone. In the case of *P. aeruginosa*, the gentamycin and piperacillin have been potentiated in the presence of the polymeric magnetic silica drug loader (Grumezescu *et al.*, 2012b).

Cellulose and their derivatives are very important since they are considered as green, natural, inexpensive, stable, biodegradable and highly biocompatibile polysugars (Habibi, 2014). Studies report a new nanocomposite prepared by co-precipitation of magnetite nanoparticles in basic aqueous solution of diethylaminoethyl cellulose (DEAE-Cellulose). Cephalosporins (cefuroxime, cefotaxime, cefoperazone, ceftriaxone, cefepime) were entrapped on the nanocoposite and used for qualitative and quantitative antimicrobial screening as drug delivery response against *E. coli* and *S. aureus* bacterial strains. All these nanosystems increased the activity of antibiotics against most tested strains (Grumezescu *et al.*, 2011a; Vlad *et al.*, 2014).

PVA is a biocompatible, biodegradable, water soluble and inexpensive polymer. It is involved in several novel nanotechnologies such as biosensors and drug targeting and delivery (Bajpai & Saini, 2006). Recently, a study highlighting the importance of PVA in the fabrication of nanocomposites based on magnetite nanoparticles has been reported. This type of nanocomposite is able to delivery cefotaxime, ciprofloxacin and gentamicin and presents a notable antimicrobial response in the case of *P. aeruginosa* for ciprofloxacin entrapped into nanocomposite, while in *S. aureus* only cefotaxime seemed to exert any effect. All reported nanocomposites present a high biocompatibility *in vitro* without affecting cell morphology, viability or cell cycle (Grumezescu *et al.*, 2012a).

Chitosan is a natural polymer obtained from crustacean shells, which offer the following properties: has a good biocompatibility; biodegradability; hydrophylicity; nontoxicity; and facilitates the transition of cellular barriers. It contains hexoses and aminohexoses that participate at the formation of bonding on the surface of nanoparticles. Chemical this type of bond is very strong and stable (Prabaharan, 2008). Nanocomposites based on chitosan and magnetite were designed to be used for drug delivery and targeting. A newly designed nanosystem based on the above mentioned materials and therapeutic agents (kanamycin sulfate and neomycin sulfate) exhibited an improved anti-microbial activity on *S. aureus* and *P. aeruginosa* tested strains. The MIC value of the antibiotics was significantly reduced in the presence of the nanosystem, thus offering new insights into developing efficient antimicrobial therapeutic strategies by lowering the amount of antibiotics yet maintaining the same activity (Grumezescu *et al.*, 2013b).

Alginates are isolated from natural sources, are biocompatible and biodragadable and find uses in biomedical applications (e.g. wound dressings and controlled release of therapeutic agents) (Rowley *et al.*, 1999). Recently, a nanocomposite based on sodium alginate and usnic acid functionalized magnetite nanoparticles has been prepared and evaluated as wound dressing. The results showed that the prepared nanocomposite exhibited a great antimicrobial activity sustained by a good biocompatibility with EA.hy926 endothelial cell line, that recommend this nanosystem as a successful candidate for improving implanted devices surfaces used in regenerative medicine (Grumezescu *et al.*, 2014a).

Polyvinylpyrrolidone (PVP) presents excellent chemical and physical properties, being a good coating agent for nanoparticles, acts as a stabilizing agent (Qiu & Mao, 2010). Limban *et al.* (2014) present a newly fabricated nanocomposite based on PVP, magnetite and benzamides with a high antimicrobial activity against *S. aureus* and *P. aeruginosa* (Limban *et al.*, 2014).

4.2 Antimicrobial Nanoshuttles

Recently, Chifiriuc *et al.* (2013) reported for the first time the importance of antibiotics functionalized magnetite nanoparticles in order to eradicate *S. aureus*, *E. coli* and *P. aeruginosa* infections. Prepared by co-precipitation, from Fe(II), Fe(III), NH_4OH and different well known antibiotics (kanamycin, erytrhomycin, polymixin, cefotaxime, ceftriaxone, amoxicillin, etc.), biocompatible magnetite nanostructures may be considered effective antibiotics carriers that are able to reduce the amount of antibiotics by improving the minimal inhibitory concentration (Chifiriuc *et al.*, 2013).

Amoxicillin functionalized magnetite nanoparticles have been reported as having a high antimicrobial activity. This nanomaterial proved to enhance the efficacy of low doses of amoxicillin against *E. coli* and *S. aureus* strains. Despite their great antimicrobial effects, these functionalized magnetite nanoparticles have no cytotoxic effects

in vitro or *in vivo*. Using am mouse model, our group has recently demonstrated that magnetite nanoparticles functionalized with antibiotics or plant-derived compounds with antimicrobial effect are well circulated through the mammalian body (Figure 4.3), offering perspectives for drug delivery and targeting applications. These results demonstrated that functionalized magnetite nanoparticles preferentially cluster in organs such as lungs and kidneys, but they are absent from other organs, as brain and liver (Grumezescu *et al.*, 2014b).

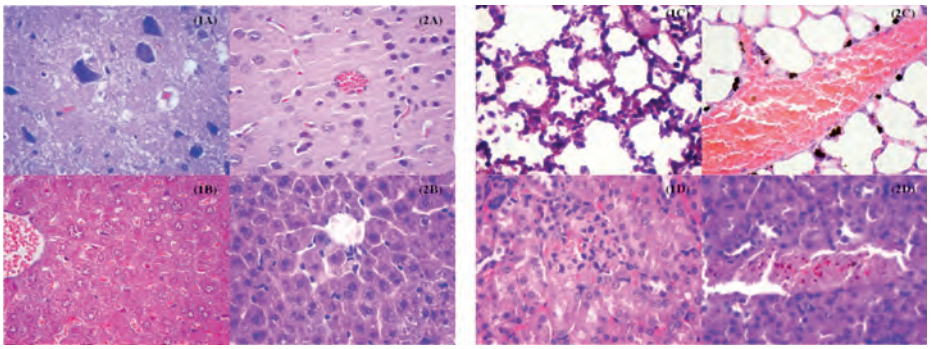

Figure 4.3: Representative transversal sections through different mice organs (A = brain, B = liver, C = lung, D = kidney) in untreated control (1) and after the treatment with the obtained magnetite nanoparticles for 48 h (2); 400× magnification (Grumezescu *et al.*, 2014).

Natural products, such as eugenol, were also used for surface functionalization of magnetite nanoparticles. Prepared by co-precipitation, with a diameter of particles less than 5 nm, this type of nanoarhitectonics highlighted a very good antimicrobial activity against *S. aureus* and *P. aeruginosa*. The reported results were compared with the effect of certain antibiotics, such as cefixime, nitrofurantoin, sufisoxaole, tetracycline, cefotaxime and carbenicillin, which are recommended for treating infections with these pathogens (Figure 4.4.) (Grumezescu *et al.*, 2013c).

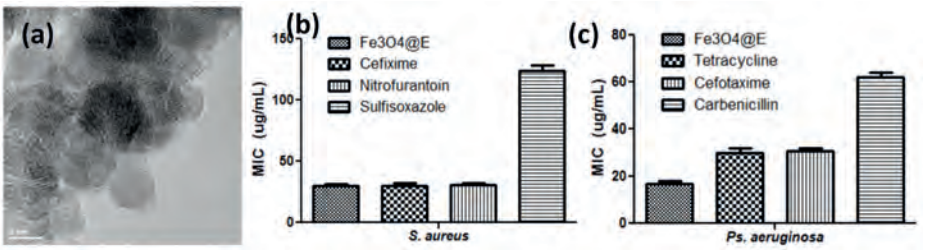

Figure 4.4: (a) Transmission electron microscopy image of eugenol functionalized magnetite nanoparticles; (b) The impact of eugenol functionalized magnetite nanoparticles on *S. aureus* viability; (c) The impact of eugenol functionalized magnetite nanoparticles on *P. aeruginosa* viability (Grumezescu *et al.*, 2013).

Usnic acid, a secondary lichen metabolite, with well-known antimicrobial activity against planktonic bacteria, was used to design novel antimicrobial nanoparticles based on magnetite. Usnic acid functionalized magnetite nanoparticles prepared by single step co-precipitation method, assure an improved delivery of the active compound to the bacterial target, even when the cells are growing in biofilms (Grumezescu *et al.*, 2013a).

All together, these data demonstrate that magnetite nanoparticles represent a powerful tool to eradicate microbial infections in combination with synthetic or natural organic compounds.

4.3 Antimicrobial Nano-modified Surfaces

4.3.1 Anti-adherent Nano-surfaces: Classical Approach

The surface of the biomaterials implanted in the human body is rapidly covered by proteinaceous conditioning film that have been shown to enhance the adherence of gram-positive cocci, gram-negative rods and *C. albicans* strains (Lazar, 2011). These microorganisms may reach the device by direct external contamination, contiguous or blood spreading, existing biofilms which are responsible for biofilm associated infections (Figure 4.5).

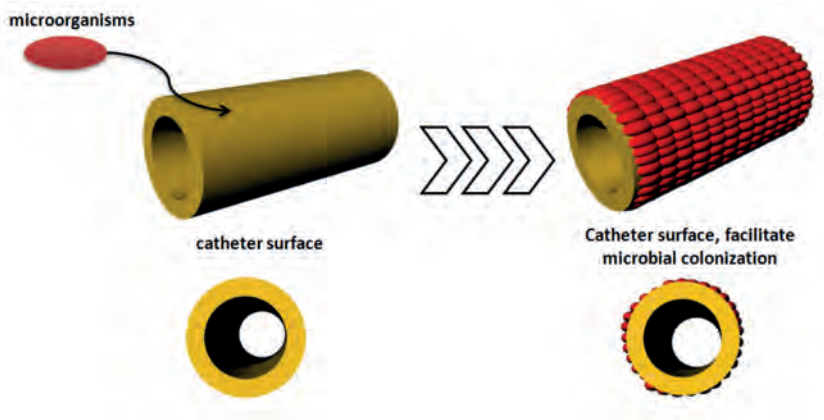

Figure 4.5: Bacteria cells colonizing a surface of a catheter.

Magnetite nanoparticles have been successfully used for coating of different medical surfaces in order to modulate microbial biofilms (Anghel & Grumezescu, 2013). Different coating techniques used are, the immersion of prosthetic device into nanofluid that is subsequently dried at room temperature (primitive/classical approach), or by laser processing techniques (advanced approach). First method was reported in the

literature as a preliminary study demonstrating proof-of-concept of the ability of magnetite nanoparticles to inhibit colonization and development of microbial biofilms (Liakos *et al.*, 2014). These results were proved by qualitative microscopic analysis. Preliminary results demonstrated a great potential of these nanoparticles functionalized with fatty acids (e.g. sodium oleate) as efficient antimicrobial coatings. Starting from this preliminary study, a series of different nanoarhitectonics based on magnetite and different therapeutic agents were designed that have proved their ability to inhibit the adherence and formation of biofilms.

Magnetite nanoparticles functionalized with *Satureja hortensis* essential oil were successfully applied as anti-adherent agent into wound dressing in order to prevent microbial contamination and biofilm formation in wounds. This study, reported by Anghel *et al.* (2013b), showed a novel formulation based on magnetite nanoparticles that were able to enhance the resistance of wound dressings fibers to fungal cell adherence and biofilm development (Figure 4.6). This type of nanosystem represents a successful alternative for inhibiting fungal adhesion on medical devices and surfaces (Anghel *et al.*, 2013b).

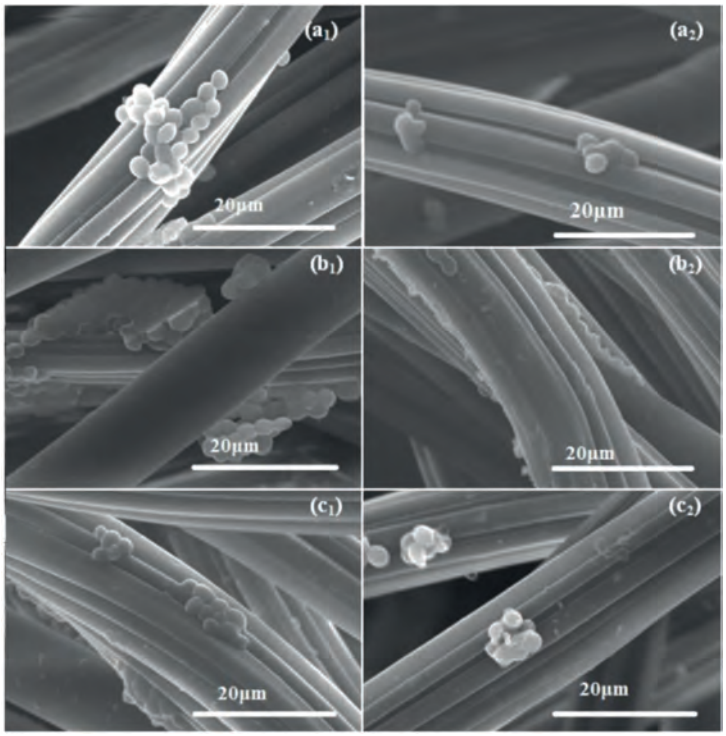

Figure 4.6: SEM (scanning electron microscopy) micrographs indicating *C. albicans* biofilm development on magnetite nanomodified surfaces (after 24 h—a_2, 48 h—b_2. and 72 h—c_2 incubation) (2500×) as compared with control (uncoated) fibers (after 24 h—a_1, 48 h—b_1 and 72 h—c_1 incubation time). *C. albicans* biofilms developed on the coated wound dressings are strongly damaged and drastically reduced (Anghel *et al.*, 2013).

Oleic acid functionalized magnetite nanoparticles have been used in order to create a thin coating for the controlled release of usnic acid. Prepared by a classical approach, the surface showed anti-biofilm activity against *S. aureus* (Grumezescu *et al.*, 2011b). Other study involved *Eugenia carryophyllata* essential oil. The same type of nanosystem has been prepared and evaluated by qualitative and quantitative assays. This nanosystem had a high efficiency against fungal biofilms (*C. albicans, C. tropicalis, C. glabrata, C. krusei*) that may develop on catheter sections. A drastic decrease of the biofilm was observed after 24–48 h (Grumezescu *et al.*, 2012a).

Bilcu *et al.* (2014) presented a novel surface based on magnetite nanoparticles and essential oils (vanilla, patchouli and ylang ylang) with improved properties against bacterial adherence and biofilm formed by clinical strains *of Staphylococcus aureus* and *Klebsiella pneumoniae* (Bilcu *et al.*, 2014). Essential oil from vanilla when coated on nanoparticles strongly inhibited both the initial adherence of *S. aureus* to the coated catheter surface and the development of a mature biofilm. The patchouli and ylang-ylang essential oils significantly inhibited the initial adherence phase of *S. aureus* biofilm development, with a decrease in the number of viable cells by at least two decades on a log scale, in comparison with the biofilm developed on the catheter samples coated only with the magnetite nanostructures. While at 48 h, the inhibitory effect was less evident, a biofilm inhibition of more than one decade on log scale was noticed compared to the uncoated catheter.

Mentha piperita essential oil was used as therapeutic agent in order to prepare anti-biofilm surfaces based on functionalized magnetite nanoparticles. This type of nanosurface prepared by a classical approach revealed good anti-adherent properties against staphylococcal adherence and biofilm development (Anghel & Grumezescu, 2013).

Similarly, *Rosmarinus officinalis* essential oil – magnetite nanoparticles nanosystem has been used to prepare a thin coating on the surface of catheter sections. Experimental assessments revealed a strong inhibition on the adherence ability and biofilm developrment of *C. albicans* and *C. tropicalis* strains (Chifiriuc *et al.*, 2012).

Recently, magnetite, limonene and eugenol were used in order to prepare antimicrobial nanosurfaces using a classical approach. The functionalized surfaces for wound dressing seem to be very useful tools for the prevention of wound microbial contamination on viable tissues. Recent results revealed that this coating affected the initial stages of biofilm formation and biofilm maturation at the three harvesting time intervals (i.e., 24 h, 48 h, and 72 h), as compared with the control, uncoated textile materials (Anghel *et al.*, 2012a).

Anghel *et al.* (2012b) reported a novel approach to coat medical surfaces by involving newly synthesized benzamides and magnetite nanostructures. Modified catheter surfaces highlighted an improved resistance to microbial colonization when compared to the uncoated ones. Results show best effect was seen on *S. aureus* and *P. aeruginosa* microbial strains (Anghel *et al.*, 2012b).

4.3.2 Anti-adherent Nano-surfaces: MAPLE Approach

Laser processing represented by MAPLE (Matrix Assisted Pulsed Laser Evaporation) technique was further used in order to prepare thin coatings for medical prosthetic devices such as catheters, voice prosthesis, titanium implants, etc. (Figure 4.7). The interest for laser processing is related to controlled topography of prepared surfaces, which can be manipulated at nano scale (Cristescu *et al.*, 2009, 2012; Mihaiescu *et al.*, 2013).

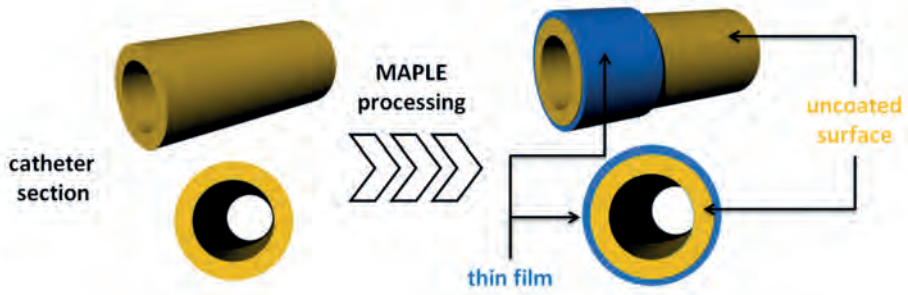

Figure 4.7: Morphology of thin films prepared by MAPLE.

Roughness, thickness and homogeneity of thin films obtained by MAPLE are aspects that can be controlled by proper handling of parameters involved in the process: weight ratio of material that is intended to be deposited and the volatile matrix, position and orientation of the substrate in the deposition chamber, the substrate temperature and laser-beam characteristics (wavelength dependence of the radiation fluence, laser pulse duration and degree of repeatability thereof, and the depth of penetration of the beam energy) (Cristescu *et al.*, 2009, 2012; Mihaiescu *et al.*, 2013).

The simplicity of the method and the possibility of obtaining coatings with controllable size, in the nanometer range along with the versatility of this technique to provide promising results for a wide variety of materials (metal, ceramic, polymeric, biological molecules) make it possible to use MAPLE for several applications in the biomedical domain ranging from coatings to generate biosensors, biocompatibilization of medical implants, bioactive coatings formulation, development of optimal surfaces with application in detection, target and treatment of disease (Anghel *et al.*, 2014; Cristescu *et al.*, 2009, 2012; Grumezescu *et al.*, 2014b; Mihaiescu *et al.*, 2013).

Thin films prepared by MAPLE provide important applications in nanomedicine, such as: drug delivery systems, tissue engineering, anti-bacterial surfaces, improved adherent surfaces, and implants with improved biocompatibility. Among these applications we will further discuss anti-bacterial surfaces and their importance in the modulation of microbial biofilms (Figure 4.8).

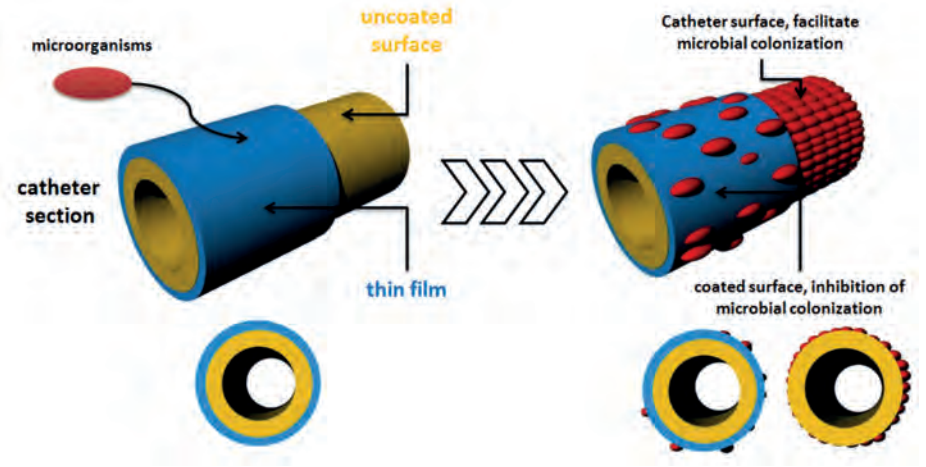

Figure 4.8: Schematic representation of biofilm development on the surface of (un)coated catheters.

Recently, a novel nanoarchitectonics prepared by laser processing, based on kanamycin functionalized magnetite nanoparticles was described. Magnetite nanostructures were prepared by co-precipitation with dimension not exceeding 5 nm. Prepared thin films were then deposited on the surface of catheters. A time dependent inhibition of *E. coli* and *S. aureus* biofilm was seen. *In vivo* data demonstrated that antibiotic functionalized magnetite nanoparticles had no toxic effects against inoculated mice (Grumezescu *et al.*, 2014b; 2014e).

Grumezescu *et al.* (2014d) reported a study that involved nanospheres thin coatings in anti-infective therapy. Titanium implants were coated by laser processing with nanosphers. First, eugenol functionalized magnetite nanoparticles were prepared by co-precipitation and were further processed to prepare nanospheres based on 3-hidroxybutyric acid-co-3-hidroxyvaleric acid, polyvinyl alcohol using solvent evaporation method (Grumezescu *et al.*, 2014c). Experiments related to modulation of microbial biofilms were conducted on *S. aureus* strains. The results revealed that the newly fabricated coatings for titanium implants present a great anti-biofilm effect. From this study, it is evident that the mature biofilm formation is altered when microbial cells are growing on the prepared thin coating.

Limban *et al.*, prepared a novel core/shell nanostructure based on magnetite and newly prepared thioureides (Limban *et al.*, 2014). The qualitative and quantitative analyses revealed a good efficiency against biofilm development. Other recent study highlighted the ability of entrapped new thiourea derivatives -magnetite nanoparticles into polymer (polyvinylpirolidone) microspheres (Figure 4.9) to achieve of an optimized anti-biofilm coating that was efficient against *S. aureus* and *P. aeruginosa* biofilms, both in the early and maturation phase (Figure 4.9) (Limban *et al.*, 2014).

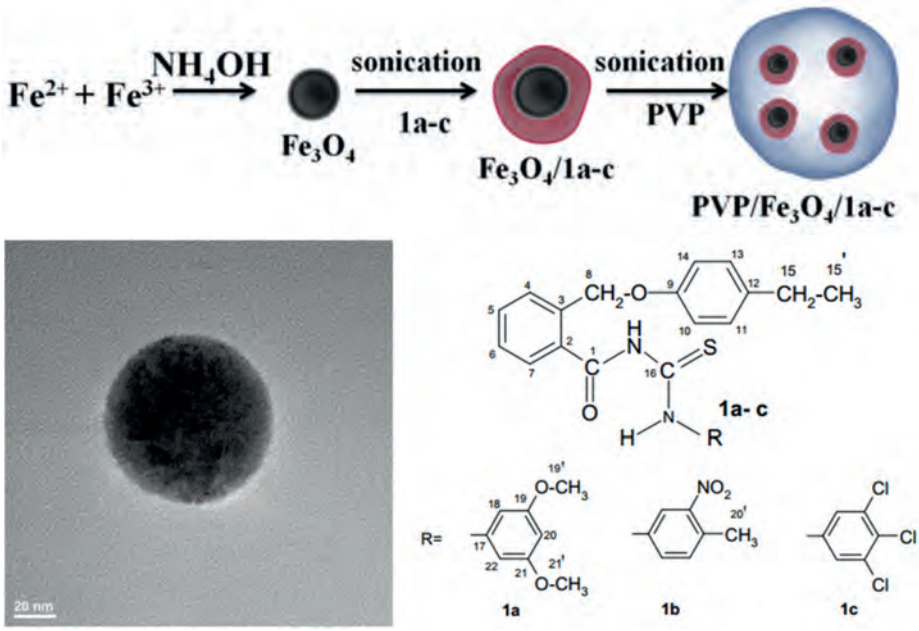

Figure 4.9: Schematic representation of PVP/Fe3O4/1a–c (new thiourea derivatives) preparation (Limban *et al.*, 2014).

Studies demonstrated that *Cinnamomum verum* used to functionalize magnetite nanoparticles, in order to prepare a thin film by MAPLE for medical purpose, revealed impressive anti-adherent properties, significantly reducing both gram-negative and gram-positive bacteria colonization (Figure 4.10). The intended application was related to the ability of antimicrobial nanoparticles organized as thin film on the surface of gastrostomy tubes to prevent colonization and biofilm formation on these prosthetic devices (Anghel *et al.*, 2014).

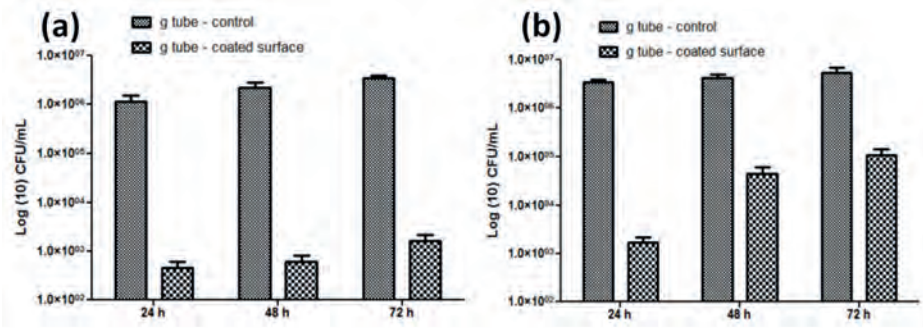

Figure 4.10: *S. aureus* (a) and *E. coli* (b) biofilms formation on ordinary (bare) and bioactive nanoparticle (nano-modified) G-tubes for 24, 48 and 72 h of incubation at 37 °C (Anghel *et al.*, 2014).

5 Conclusions and Perspectives

Even if we are living in an era of antibiotics, infectious diseases represent one of main causes of death and severe debilities, significantly affecting humans life. Because of the rapid spread of antimicrobial resistance phenotype, alternative anti-infectious strategies are urgently needed. Since there has been little to no progress in the development of new antibiotics, clinicians have to think outside of the box and take more precautions. Although this strategy proved some improvements in the infection control in several hospitals and health-care facilities, most community and healthcare associated infectious diseases remain uncontrolled. In the past years, along with clinicians, researchers struggled in finding alternative strategies to combat severe infections, especially those associated with resistant pathogens. In the actual context, a promising approach seems to rely on finding the best method to manipulate microbial behavior and virulence in order to limit the harmful action of pathogenic bacteria.

Many natural and synthetic molecules are able to be recognized and utilized by pathogenic bacteria and most of them have the potential to be used in the development of alternative antimicrobial approaches by controlling microbial behavior and virulence. Even though the molecular mechanisms of these molecules are yet to be elucidated, the fact that they function within the bacterial cells may explain the change in bacteria virulence and behavior under different clinical conditions. Along with the research and clinical-related role, this molecular regulation and signaling has a great potential for application. The practical potential resides in the ability of natural compounds to be used in handling severe bacterial infections, because they proved to act as great regulators for bacteria virulence and communication. Since these phenotypes usually do not affect bacterial survival, these modulators may be efficiently used to control infections without taking the risk of developing microbial resistance. In recent years, many pharmaceutical companies have focused their activity in developing alternative antimicrobial therapeutic approaches based on natural or synthetic molecules able to modulate virulence and cell-to-cell communication in order to disrupt the phenotypes involved in the pathogenicity of harmful bacteria. Furthermore, the current technological progress allowed the development of specific vectorizing agents, which have an important role of stabilizing, targeting, controlling the release and improving the activity of novel and alternative antimicrobial compounds. These functionalized nanosized molecular shuttles, composed of different components, in several forms, have proved to have a great antimicrobial effect, while maintaining safety for the human use. Although the current knowledge is still limited regarding these new approaches aiming to resolve and combat severe infections, they represent a research priority in the biomedical field and researchers agree that they represent the base of alternative, ecologic approaches in fighting resistant and persistent pathogens.

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