New antiinfectious strategy based on antimicrobial and quorum sensing inhibitors from vegetal extracts and propolis

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The high level of multidrug resistance resulted in the prediction that we are approaching to the preantibiotic era and determined a new perspective into the research field of new antipathogenic drugs, without any selection pressure and resistance inducing or other side effects. This new kind of antiinfectious drugs are based on the discovery that the success of pathogens is based on their ability to sense the environment, respectively all signals concerning their own density, but also produced by different cells of the host organism. So, they must be able to receive signals and to respond to them, activating their virulence genes and tending to avoid or inactivate the host unspecific and specific defence mechanisms and to use the host factors for their own benefit. The increased cellular density of a microbial community or biofilm and the consequent accumulation of cell-to-cell signalling or quorum sensing (QS) molecules is contributing to a better adaptation of the metabolic activity of microbial cells, including their tolerance to antimicrobials and synthesis of virulence factors, synchronizing their different genes expression in a particular cell density, according to each step of the infectious process [1,2,3]. It is proved that a lot of organisms, such as commensal bacteria (and probiotics too), algae, lichens, plants and animals synthesise some molecules called QS inhibitors (QSI) [4, 5], as a defense mechanism, able to interfere with the QS mechanism of pathogens and reducing their injuriousness, as an expression of their coevolution and coadaptability. In recent years, following the general trend of using different drugs provided by nature, plant and bee products have became such natural resources, searched and promoted for a wide range of harmless therapeutic applications. Plants are well known for their content in different antimicrobials, including QSI [6]; more recently it was shown that honney bees are able to protect them against pathogens producing antimicrobials and QSI. These molecules have double origine: from the bees organism and from the visited plants, and their chemical composition varies according to geographical area and plant source; among these, propolis is one of the most valuable bee product, due to its rich content in nutrients and products with prophylactic and therapeutic effects [7, 8]. The interkingdom signalling is a relativelly recent field of research, very promissing for a new perspective about an antiinfectious therapy menagement. In this paper we review the main pathways for fight against pathogens and their virulence factors by natural antimicrobials targeting the QS circuits, focusing on vegetal and honey bees bioactive molecules; it is a new target and an intelligent strategy, without any risk of inducing resistance [9].

Keywords Bacterial antibioresistance and virulence; Biofilm tolerance, QS inhibitors, Antipathogenic strategy, Vegetal extracts, Propolis.

1. Introduction

During millions of years antibiotics and antibiotic resistance genes have co-evolved slowly. The overuse and misuse of antibiotics, these paradoxal drugs, beneficial and also detrimental, induced the present high level of antibioresistance also augmented by the overuse of other antimicrobials (antiseptics, disinfectants, biocides), confrunting mainly the medical world, but even other sectors (agriculture, food industry, drinking water, fresh waters,) with an indirect impact on public health. Although antibiotics have drastically reduced illness and death from infectious diseases along the decades after their discovery and availability for therapeutical use, bacteria have exhibited a remarkable capacity to quickly become more and more resistant to antibiotics [10]. The widespread use antibiotics has evolutionary and ecological consequences; such antibiotic pollutants have non-target effects, raising the general rates of mutation, recombination, and lateral gene transfer in all the microbiome, and simultaneously providing the selective force to fix such changes. This has the consequence of recruiting more genes into the *resistome* and *mobilome*, and of increasing the overlap between these two components of microbial genomes.The human use and environmental release of antibiotics is having second order effects on the microbial world, because these small molecules act as drivers of bacterial evolution. Continued pollution with both xenogenetic elements and the selective agents that fix such elements in populations has potentially adverse consequences for human welfare [11].

The antibioresistance (AR) has been declared by ECDC (*European Center for Disease Control*) as one of the four major public health problems. The increasing occurrence of multiresistant pathogenic bacterial strains has gradually rendered traditional antibiotics ineffective. The prognosis is worsened by the formation of bacterial *biofilms* on cellular substrata, intact or damaged and also on biomaterials used in medicine, due to their behavioural resistance, recalcitrance or tolerance, even if the component cells tested in suspension (by the standard method) could be susceptible to some antibiotics.

In this context, the research for finding new preventive or therapeutic antiinfectious strategies become a top priority at international level. The necessity of focusing and harmonisation of the international scientific research efforts to find

new and efficient alternatives for the treatment of chronic infections with multi-resistant and biofilm-forming microorganisms was figured out by experts at international meetings during the last decade. In addition, the proximity of cells in biofilms increases the possibility of gene transfer between the species, converting a previously avirulent or low virulent susceptible commensal organism to a high virulent and resistant pathogen. Some of these genes may be advantageous for bacteria, enhancing the survival under unfavorable conditions such as nutritional starvation and high cell density, two key characteristics of biofilm physiology. Hence, plasmids including those that confer drug resistance and provide enzymes expand the nutritional ability and virulence determinants of cells, increasing their fitness property. The enhanced efficiency of gene transfer in biofilms induces enhanced stability and also facilitates the spread of AR in polispecific/policlonal biofilms.

The problem of development of effective strategies for the prevention and treatment of infections with multiresistant and biofilm forming bacteria is still less studied, despite their great applicative potential in biomedicine and other fields (food industry, drinking water system, agriculture). Moreover, the interactions between the various species in mixed infections are synergistic in that the presence of one microorganism generates a niche for other pathogenic microorganisms (as in environmental microbial communities), predisposing the host to colonization by a second pathogen, a multispecific biofilm being much more resistant to antibiotherapy and other stress conditions. At present, the quite often used antibiotherapy (more or less necessary) for fighting against microbial infections during the last decades exerted a high selective pressure and conducted to the actual level of antibioresistance. The emergence of multidrug-resistance phenomenon caused increasing concern for finding new antimicrobial agents and new antiinfectious therapeutical strategies. Moreover, due to the modern and quick circulation of people and products, the increased antibiotic resistance is thus a global problem. The high level of multidrug resistance resulted in the prediction that we are approaching to the preantibiotic era and determined a new perspective into the research field of antipathogenic drugs, without any selection pressure and resistance inducing or other side effects. Considering all of these and following the general trend of using resources provided by nature, in recent years, vegetal extracts and bee products too have became an important source for bioactive factors, promoted as harmless therapeutic drugs. This new kind of antiinfectious drugs are based on the discovery that the pathogens are able to sense the environment, respectively all signals concerning their own density, but also produced by different cells of the host organism. So, they must be able to receive signals and to respond to them, activating their virulence genes and tending to avoid or inactivate the host unspecific and specific defence mechanisms and to use the host factors for their own benefit. The increased cellular density of a microbial community or biofilm and the consequent accumulation of cell-to-cell signalling or quorum sensing (QS) molecules is contributing to a better adaptation of the metabolic activity of microbial cells, including their tolerance to antimicrobials and synthesis of virulence factors, synchronizing their different genes expression in a particular cell density, according to each step of the infectious process [1,2,3]. During the last 20 years, the search for new large spectrum antimicrobials has led to very few new classes of molecules, even the rising leveles of resistance in pathogenic microorganisms create an urgent need for new antimicrobial agents, not affected by the resistance mechanisms already present in bacterial population [23]. Considering the fact that the great majority of infections (conform to public announcements stated) are biofilm associated infections (60-85%) caused mainly by opportunistic pathogens and that the virulence features are expressed in high density populations or biofilms, targeting the communication ways or circuits seems to be an inteligent strategy to fight pathogens. It is proved that a lot of organisms, such as commensal bacteria (and probiotics too), algae, lichens, plants and animals synthesise some molecules called QS inhibitors (QSI) [4, 5], as a defense mechanism, able to interfere with the QS mechanism of pathogens and reducing their injuriousness, as an expression of their coevolution and coadaptability. In recent years, plant and bee products have been searched for the presence of QSI and other bioactive factors. Plants are well known for their content in different antimicrobials, including QSI [6]; more recently it was shown that honney bees too are able to protect them against pathogens producing some antimicrobials and QSI too; the bee products have double origine: from the bees organism and from the visited plants, and their chemical composition varies according to geographical area and plant source; among these, propolis is one of the most valuable bee product, due to its rich content in nutrients and products with prophylactic and therapeutic effects [7, 8].

The aim of this paper is to review the main pathways for fight against pathogens and their virulence factors by natural antimicrobials targeting the QS circuits, focusing on vegetal and honey bees molecules; it is a new target and an intelligent strategy, without the risk of inducing resistance [9].

2. Microbial Biofilms and Quorum Sensing Mechanism

After the first reports at the beginning of the last century (ZoBell, 1933) about bacterial communities on all submerged surfaces only in 1978, Costerton and his team described the microbial biofilms and emphasized the very significant role of these adherent microorganisms in human infectious diseases and many other processes [12-16]. Biofilms may develop on living or non-living surfaces, and represent a prevalent mode of microbial lifestyle in natural, industrial and hospital settings. Bacteria possess specialized surface structures called *adhesins*, able to interact stereospecifically with *receptors* on the host cell membrane, in a manner analogous with the interaction antigen-antibody. The adhesins are composed of polysaccharydes and proteins and form the biofilm matrix. Surface proteins play a critical role in the

interaction between cells and their environment, as they take part not only in adhesion process, but even in processes like signaling and transport. In pathogenic microorganisms, they can also participate in virulence or cytotoxicity. As these proteins have the highest chances to be recognized by the immune system, they are often the targets for the discovery of new vaccines. In the last years, a novel second-generation approach has been developed (after the first one – biochemical identification), consisting of the digestion of live, intact cells with proteases, so that surface-exposed moieties (i.e. the "surfome" of a cell) are "shaved" and analyzed by LC/MS [17].

Adherence to the substratum is the initial stage of biofilm formation; in order to form a biofilm the microorganisms must adhere to a substratum, and then they will grow and survive even if the environmental conditions are not so favorable (pH, redox potential, low concentration of nutrients). The biofilm formation either in natural or industrial systems is now seen as an adaptation and survival strategy of microorganisms, the biofilm cells being protected from all stress conditions, including all kind of antimicrobials. A widely accepted definition of a biofilm is: a microbial community composed of cells irreversibly attached to a substratum, to an interface or between them, embedded in a matrix of extracellular polymeric substances secreted by them selves and which present a modified phenotype concerning the growth rate and gene transcription [18]. The architecture of a mature biofilm reveals *citadels*-like structures, presenting *mushrooms*, *columns* and *pillars*-like surface relief. Between the matrix encased cells there are spatiotemporal relationships, a synergistic, metabolic cooperation; a microbial biofilm is now considered a primitive form of cellular differentiation, with a primitive "circulatory system", "homeostazy", "integrality", similar with the eukaryotic tissues in their intercellular cooperation [14]. So, the physiological properties of the biofilm cells are different from their counterpart, the free or floatting cells. Firstly, the growth rate is less intense, which is very important for their new behaviour in these conditions. So, in a mature biofilm, more volume is occupied by the loosely organized glycocalix matrix (75-95%). In most cases, the base of the biofilm is a layer with thickness up 10 to 50 μm, composed of a sticky mix of polysaccarides, other polymeric substances produced by bacteria and watter. Biofilms are permeated at all levels by a network of chanels through which watter, nutrients, bacterial metabolites, enzymes and oxygen move to and from, with gradients of chemicals and ions between microzones providing the power to shunt the substances around the the biofilms [19]. The biofilm's matrix, which is also referred to as *slime* is a reticular structure generally composed of water (95-99%), polysaccharides, extracellular DNA, proteins and minerals. The term of *slime* was used by Christensen et al. (1982) and it meens the glycocalix produced by the strongly adherent strains of *Staphylococcus epidermidis* isolated from the infected surface of medical implants which produce a polysaccharide intercellular adhesin (PIA) essential for cell-to-cell adhesion and subsequent biofilm formation [20]; today the terme is also used for other species.

The majority of bacteria live not planktonically, but as residents of sessile biofilm communities, formed to both biological and nonbiological surfaces. In medical "ecosystems", microbial adhesion leads to biofilm formation on various surfaces, natural (teguments and mucosa, intact or damaged, teeth, endothelial cells) or artificial (different prosthetic materials) and is a precondition to pathogenesis of many bacterial infections, difficult to treat, due to the different behavior of cells in biofilms. Biofilm formation is implicated in many chronic disease states because this mode of growth promotes survival by increasing community recalcitrance to clearance by host immune effectors and therapeutic antimicrobials [21-23].

As any form of life, the biofilm development (progression from the reversibly adherent cells, to irreversibly adherent cells, multiplication and colonization of substratum, biofilm maturation) is cyclic and interrupted by a desaggregation process, clusters of isoleted cells being released from the mature and thick biofilm, mainly due the physical process of fluids flow and to the autolysis process of the deep starved cells [3]. Moreover, enzymes capable of cleaving essential components of the biofilm matrix (e.g. polysaccharides or extracellular DNA), and thus weakening the biofilm architecture have been identified, so bacteria also have mechanisms to dissolve their biofilm and return to planktonic lifestyle [24]. Biofilms are considered a real social arrangement, with survival, adaptative, protective value which are the hallmarks of biofilms, different from the free, planktonic cells, which are less efficient, produced only for dissemination and colonization of new environments; if for bacteria this process is saviour and reveals their great adaptation capacity, for humans can represent a high risk of serial contamination of drinking water or of food products, or dissemination of an infection; in this last case cells above the shield of slime might be expandable, acting as a mean analogous to metastatic cancer cells [25].

The capacity of bacterial cells to behave collectively inside the biofilm, as a group is the result of complex intra- and inter-cellular signaling regulated by quorum-sensing and response (QS) mechanism, ubiquitous in bacteria, involved in the regulation of very diverse and complex physiological processes, including expression of virulence properties, depending on cellular density. At the molecular level, the "language" used for this intercellular signalling is based on small, self-generated signal molecules known as QS molecules with different chemical structures (N-acyl-homoserine lactones (AHL) and derivatives in Gram-negatives, octapeptides, amino acids in Gram-positive bacteria, AI-2 molecules common to Gram positive and Gram negative bacteria, acyclic sesquiterpene alcohol called farnesol and tyrosol - an aromatic alcohol in *C. albicans*) [26, 27, 28].

The understanding of the mechanism of crosstalk between the bacterial cells, and between bacteria and host cells may contribute to the elaboration of an efficient strategy for controlling the severity of biofilm associated infections, based on the disruption of the intercellular communication. This strategy is termed „anti-pathogenic", because it is not

based on interference with microbial growth, reducing only the virulence factors expression and by consequence the microbial pathogenicity. There have been already elaborated some therapeutic anti-infective strategies based on the inhibition of QS molecules generation in Gram-negative bacteria, consisting in: i) inhibition of AHL synthesis [29]; ii) inhibition of the AHL signal dissemination by the use of some bacterial enzymes called lactonases or quorum-quenching enzymes, or by non-enzymatic, alkaline hydrolysis or using of some microorganisms (e.g. *Variovorax paradoxus*) which can use AHL as a unique source of C and N; iii) inhibition of the signal reception by using competitive molecules (with a homologous structure to that of bacterial QS molecules) or uncompetitive (with a different chemical structure) capable of interfering with the AHL binding to the sensor protein or with the signal transmission to the regulator, transcriptional protein [26]; iv) preventing the signal from being perceived by the bacteria, by either blockage or destruction of the membrane sensor or receptor protein by a LuxR homologue.

The signalling molecules allow to bacterial cells the environment monitoring and a modified gene expression and in the same time, the acquirement of a competitive advantage important for survival and dissemination in natural high competitive environments, such as the oral cavity or intestinal tract [26, 3]. Alternatively, the presence of one microbial species cells may generate a niche in the host that suppresses the colonization with another species. This form of interaction is called "microbial interference" and it is the basis for the anti-infectious barrier effect exhibited by normal microbiota and for the use of probiotics. The understanding of the molecular mechanisms of the cross interactions in polymicrobial biofilms associated infectious in humans will open new perspectives on prophylactic and therapeutical strategies targeting especially polymicrobial biofilms, very difficult to be treated by antibiotics. QS systems discovery allowed to open new prospects for treatment management of bacterial infections by finding the inhibitors of bacterial QS mechanism, represented by natural or synthetic compounds [28, 30].

The recent advances made in highlighting the molecular genetic basis of biofilm development and the deciphering of the inter–species communication by QS systems regulating bacterial virulence has afforded a novel opportunity to control infectious bacteria, without interfering with their growth, leading to the development of new preventive and therapeutical strategies, based on biofilm-controlling agents, exhibiting the advantage of not being growth inhibitors; so, they do not act as selection factors for resistance genes, constituting thus an ecological strategy, but act as inhibitors of the biofilm formation and by disrupting the biofilm'cells connection, rendering the bacterial cells susceptibility to usual therapeutical doses of antibiotics.

3. Consequences of Biofilm Formation

3.1. Virulence genes expression and biofilm tolerance to antimicrobials

The intercellular communication between the biofilm cells and the increased concentration of signal molecules exert a control on a lot of bacterial processes depending on the cellular density and environment changes, such as: mobility, sporulation, biofilm development and synthesis of virulence factors (exopolysaccharides, enzymes, siderophores, toxines etc.). By employing the QS system to control expression of its virulence factors (a lot of them being antigenic determinants), *P. aeruginosa* is able to operate in a stealthy manner until a certain cell density is reached, where the QS systems become activated. Upon activation of the QS systems, a coordinated release of tissue-damaging and immune defense-degrading virulence factors takes place. For instance it was recently documented that the QS-controlled virulence factor rhamnolipid (also known as heat-stable hemolysin) destroys polymorphonuclear leukocytes (PMNs) by lytic necrosis; besides lysing neutrophils and macrophages, rhamnolipid has also been reported to impair chemotaxis of neutrophils [31].

One of the most important consequences of biofilm formation is that the biofilm cells are more resistant to both host defenses and conventional doses of antibiotics and biocides, even the same cells tested by the standard method for M.I.C. determination (with cells in suspension) are susceptible to antimicrobials action; this resistance is named behavioural resistance or recalcitrance [21] or, more recently called *tolerance* to antimicrobials [32, 33, 3]. Tolerance is defined as the ability to survive killing by bactericidal factors without necessarily expressing a genetic resistance mechanism. The molecular basis of tolerance is still not completely understood, but it is especially significant in survival of bacterial biofilms. It has recently been found that „persister cells' are largely responsible for the high tolerance of bacterial biofilms to antimicrobials. In a variety of bacterial species examined, the level of "persisters" increased with the density of the culture, reaching ~1% in stationary phase as well as in biofilms of *Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus* [34, 35]. A number of mechanisms accounting for the increased recalcitrance / tolerance of biofilms include: 1) failure of the antibiotic agent to penetrate the extracellular matrix which acts as a diffusion barrier for large molecules (some antibiotics, antibodies, phagocytes) or as an ions exchanging resin; in the same time the matrix can accumulate waste products (which enable continuation of the log phase); 2) accumulation of antibiotic degrading enzymes; 3) a different pattern of gene expression occurs in irreversibly adherent bacterial cells to different substrata (40-60 % of prokaryotic genome being up or down regulated) [36] (e.g. under-expression of porin genes as Omp F that mediating the internalization of the majority of hydrophilic antibiotics, over- expression of efflux pumps actively pumping out the antibiotics, the activation of some virulence genes); 4) the cells experience nutrient limitation in the depth of a mature biofilm and, therefore, a slowgrowing or starved state can

be present or, the nongrowing cells are not highly susceptible to antimicrobial agents; this genomic plasticity of biofilm is conferring high levels of diversity, so any given antibiotic cannot kill all of the cells in the community and certain cells will persist and repopulate the surface - (*persisters* theory) [16]; 5) occurrence of genetic changes such as: a) *mutations*; cells with a high rate of mutations (*hypermutators*) were isolated from biofilms, probably selected by different stress conditions, with an adaptive role [36]; b) *gene transfer* (including R and virulence plasmids) facilitated by the proximity of the cells and leading to an acquired resistance in the initially susceptible bacterial cells of multiclonal or multispecies communities. Even the "persisters' were firstly mentioned since 1944 by Joseph Bigger (who noticed that penicillin did not sterilize a culture of *Staphylococcus aureus)*, but their presence was understood much more later. The surviving *persister* cells, may be small in number, i.e. less than 100 persisters out of 2.5x107 cells after exposure to penicillin. Opposite to resistant mutants, persisters are phenotypic variants of the wild type that after reinoculation are promoting a culture with a similar amount of persister cells. There are data pleading for the fact that persisters are not signifying a particular stage in the cell cycle and that they are not produced in response to antibiotics, but there are rare non-growing cells preexisting in a bacterial population. Bacterial populations produce *persister cells that neither grow nor die in the presence of the bactericidal agents, but they are exhibiting multidrug tolerance* (MDT) [35]. It can summarize that the tolerance to antibiotics of both some planktonic cells and biofilm populations has been suggested to be due especially to slow growth rates.

In 2000, the Center for Disease Control and Prevention (U.S.A.) cited biofilm-associated diseases as one of the seven major healthcare safety challenges facing the medical community. The single most important aspect of these challenges is the remarkably increased antibiotic non-inherited resistance or *recalcitrance* / *tolerance* of the biofilm's cells. The biofilm phenotype can reduce antimicrobial susceptibility and increase tolerance up to 1000- 4000 times, evidently decreasing the antimicrobial efficiency and leading to clinical therapeutical failures. The minimal inhibitory concentration (MIC), determined by standard assay on free cells in suspension is clearly in contrast with the minimal biofilm eradication concentration (MBEC) [25].

3.2. Interkingdom signalling and natural QS inhibitors of different origins

It is well known that the success of pathogens is based on their ability to sense the environment [37], respectively all signals concerning their own density, but also produced by different cells of the host organism. So, they must be able to receive signals and to respond to them, activating their virulence genes and tending to avoid or inactivate the host unspecific and specific defence mechanisms and to use the host factors for their own benefit. The increased cellular density of a microbial community or biofilm and the consequent accumulation of QS molecules is contributing to a better adaptation of the metabolic activity of microbial cells, including their tolerance to antimicrobials and synthesis of virulence factors, synchronizing their different genes expression in a particular cell density, according to each step of the infectious process [1,2,3]. It is proved that all living organisms, such as commensal bacteria (and probiotics too), algae, lichens, plants and animals synthesise some molecules called QS inhibitors (QSI) [4, 5] for their antiinfectious protection, able to interfere with the QS of pathogens and reducing their injuriousness, as an expression of their coevolution and coadaptability.

Intraspecific microbial communication, based on QS mechanism is important for the cell's phenotype/behaviour regulation, adequate to environment; but even the recognition of an universal "language", which allows: the interspecific communication between bacterial species; the interkingdom signaling - between bacteria and eukaryotic cells: microbial or host cells. This real "language" assure the succes of a pathogen, dependent on its ability "to sense" the environment and to modulate the expression of the genes encoding factors required for the colonization of a new habitat [34]; AI-2 is un universal interspecies signal ("cross talk") has been identified at 55 pathogenic species Gram positive and negative and is dependent of cellular density, having an important role in intensively colonizated situs: oral cavity, intestine; these processes are important in multispecific biofilms architecture, but also in infection and disease; or, microbial adherence and biofilm formation comprise ~ 60-85% of total infection cases, on tissues/medical devices. The communication and relative densities of opportunistic *Candida albicans* and commensal bacteria decides the host condition of health or disease [26, 27]. Another QS membrane receptor is the histidine kinase sensor QseC which is conserved in several bacterial species. It controls the expression of virulence genes in response to AIs, but also to the host hormone epinephrine; Sperandio and co-workers, reported the screening of a large number of molecules as QseC antagonist using an *E. coli* reporter gene assay and selected a potent inhibitor of of QseC in *EHEC strains* [38]. In mamalian serum and in human epithelial cells were identified *lactonases* – like molecules or *quorum quenching* enzymes, as a component of innate defense mechanisms [39], but even germinated seeds – contains some similar *QS inhibitors* – with antiinfectious effect [40]. The paraoxonases (PONs) constitute a group of lactonases (a serum A-esterase able to hydrolyse the toxic oxone metabolite of parathion, hence paraoxon) ubiquitously expressed in human tissues, with 3 members, named PON1, PON2, PON3; in humans, PON1 and PON3 genes are mainly expressed in the liver and kidney and their protein products are found in the circulation bound to high-density lipoprotein (HDL) and PON2 on various tissues. Generally there are a few clinical studies in this field; for example, the relationship between circulating PON levels and the severity of various chronic infectious diseases has yet to be established. An interesting approach, taking into account that PON enzymes are proposed to be antiatherogenic, could investigate whether

alterations in their circulating levels can provide a link between periodontal disease and the increased risk of arteriosclerosis and associated pathology [41].

To adapt to the very hostile environment of a host organism, pathogens use several sensing and signal transduction pathways which could represent targets for new inhibitors. A large variety of virulence features are now the targets for this new type of antimicrobials. Diverse bacterial functions, essential for pathogenicty offer a large number of potential targets. Some virulence targets, common for several bacterial species among the most frequently isolated in clinique (enterobacteria, staphylococci and *Pseudomonas sp*.), have led to the identification of antivirulence products or antipathogenic drugs, with a large antimicrobial spectrum. QS controls the expression of many virulence factors; for example, the bacterial adherence and colonization could be inhibited using new inhibitors of *E.coli* pili synthesis, using *sortases* or specific inhibitors of type III secretion systems (TTSS); among the last category, small molecules active against enterobacteria and intracellular bacteria can be promising antivirulence drugs. Sortases allow the secretion and anchoring of many cell-wall proteins, as adhesins and pili; due to the high degree of conservation in Gram-positive bacteria, sortases have been considered a good pharmacological antivirulence target for these pathogens; these enzymes act by the cleavage of the protein LPXTG motif of the sorting signal and the inhibitors of sortase such as peptidomimetic molecules (diarylacrylonitriles) have a potent inhibitory effect against *S. aureus*, but even flavonols, such as morin (can be isolated from Maclura pomifera - osage orange, Maclura tinctoria - old fustic and from leaves of Psidium guajava - common guava), could also inhibit *S. aureus* sortase. Another possible target is the siderophore synthesis or heme regulation, the iron metabolism being essential for pathogenic bacteria in the host. Another way to efficiently fight against pathogens is the inhibition of bacterial resistance to host defence mechanisms, using small molecules inhibiting some metabolic ways involved in the defence of against the oxidoreduction stresses or cationic effectors of the innate immunity (defensins) [38].

It has been shown that some terrestrial and marine organisms have evolved a system of specific molecules with AHL-antagonistic activity capable of interfering with the bacterial QS system in a possible prevention of colonization [28]. Due to the importance of biofilm formation at medical, ecological, and economic level, in the last ten years an extensive research on this phenomenon has been done [69]. A very attractive alternative to the antibiotherapy and in the same time an ecological strategy for fighting against the biofilm associated infections is the inhibition of intercellular signalling by natural QS inhibitors (QSI) secreted by other organisms, microbial, vegetal and animal too [3]. In recent years, vegetal extracts and bee products too have became an important source for different bioactive factors. Propolis is one of the strongest challenges to nutritionists and medical world due to the recognition of its high biological value and both prophylactic and therapeutic effects.

For the QS and and biofilm inhibitors local treatment seems the obvious indication, permitting a quick assessment of efficiency in the host. For the antivirulence molecules addressing factors necessary for bacteria to avoid distruction by the immune system, they could be used to treat or prevent bacteremia and the inhibition of the interaction with host immune defenses needs to be validated in the human host. Concerning the antivirulence compounds affecting the cell wall composition, the bacteria are more sensitive to antibiotics, and so combination therapy with current antibiotics could be envisioned [38].

3.3. Vegetal extracts, fractions and their effects

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens. These plant substances are classified as phytoanticipins, which are compounds that are present constitutively, or phytoalexins, whose levels increase strongly in response to microbial invasion. In several well-documented cases, mutant plants that lack the ability to produce a particular phytoalexin had considerably higher levels of sensitivity to microbial pathogens [42]. Plant compounds are routinely classified as "antimicrobial" on the basis of susceptibility tests that produce MICs in the range of 100 to 1,000 μg/ml, orders of magnitude weaker than those of typical antibiotics produced by bacteria and fungi (MICs, 0.01 to 10 μg/ml). A compound that is synthesized in response to pathogen invasion and is required to protect the plant from a pathogen but that shows little activity in an *in vitro* susceptibility test is not necessarily an antimicrobial. Such a substance might have a regulatory function, indirectly increasing the level of resistance of the plant. This analysis suggests that we lack a solid rationale for making a functional assignment for the vast majority of plant compounds that have been classified as antimicrobials. One helpful clue regarding the possible function of plant secondary metabolites is that these compounds often show considerable activity against Gram-positive bacteria but not against Gram-negative species or yeast, because both of these groups of pathogens have evolved significant permeability barriers; for example, in Gram-negative species, an outer membrane is a fairly effective barrier for amphipathic compounds, and a set of multidrug resistance pumps (MDRs) extrudes amphipathic toxins across the outer membrane; similarly, in the yeast *Saccharomyces cerevisiae*, the presence of ergosterol, which decreases permeability, combined with a set of broad-specificity MDRs also provides an effective barrier. By contrast, the single membrane of Gram-positive bacteria is considerably more accessible to permeation by amphipathic toxins, and MDRs provide limited protection. These facts could explain that only several Gram-positive bacteria invade plants, but the majority of plant pathogens are belonging to Gram-negative bacteria or yeast [42]. A lot of plants are well known for their antimicrobial activity or other beneficial effects on humans: antiinflammatory, immunomodulatory, anti–oxidative effect; for example, this anti-

oxidative and the consequent antitumoral effect is well known for the green tee and lycopene as other carotenoids too; sometimes, the effect is combined: anti-oxidative and antimicrobial; e.g. lycopene, a natural antioxidant, may be beneficial for treatment of *H. pylori*-induced gastric diseases associated with oxidative DNA damage [43]. Although the potential of vegetal extracts to be used as therapeutical remedies is known since longtime, their use is still empirical, but the problem of antibiotic resistance as well as the negative impact of the antibiotics discharged in the external environment on the ecological balance have reinforced the studies concerning the characterization of the chemical structures of vegetal products and the active doses, aiming to understand their specific mechanisms of action. Moreover, they can present synergistic effects, and can represent alternatice or complementary solutions for an efficient antiinfectious treatment. In the strategy of QS blocking there are also used inhibitors secreted by higher organisms, such as halogenated furanones secreted by the Australian marine red alga *Delisea pulchra* which inhibits bacterial QS, preventing surface colonization and biofilm formation, as well as the subsequent biofouling process [44]. One drawback, however, is that the halogenated furanone derivatives are probably too toxic for humans. An alternative to avoid side-effects related to toxicity is to search for biofilm-controlling compounds among herbal products. Recent studies have shown that many photosynthetic organisms, either aquatic or terrestrial plants have developed as a defence mechanism against bacterial colonization, some metabolites which inhibit the intercellular signalling, called *Quorum sensing inhibitors (QSI)* (ex. *Usnea barbata, Nimphaea sp., Allium sativum, Capsicum annuum, Pisum sativum* etc.) [45]. It is important to mention that several new QSI were shown to be active *in vivo* too, validating the notion that targeting QS has potential for the antimicrobial drug development. For example, from a well known plant with antimicrobial activity - garlic (*Allium sativum*), by a bioassay-directed purification procedure, a sulfur-containing compound named *ajoene (*4,5,9-trithiadodeca-1,6,11-triene 9-oxide) was extracted and identified as a component with QSI activity; when garlic is crushed, ajoene and several other organosulfides are produced as degradation products of allicin (diallyl thiosulfinate). Ajoene has been reported to display conventional antimicrobial activities against a number of Gram-positive bacteria and the Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Xanthomonas maltophilia* but not *P. aeruginosa*. To further exploit the QSI activity *in vitro* and *in vivo*, Jakobsen and co-workers employed chemically synthesized ajoene. The *in vitro* experiments showed significant inhibition of a subclass of QS-regulated *P. aeruginosa* genes and a significant synergistic action with tobramycin with respect to the reduction of viability of biofilm cells. Furthermore, a mouse model of pulmonary infection was employed to demonstrate the antimicrobial effect of ajoene on *P. aeruginosa* infections [31].

The potential of vegetal extracts (*Ocimum basilicum, Mentha piperita*, *Salvia officinalis, Eugenia carryophyllata*) for eradication and prevention of microbial biofilms is gaining increasing interest due to their relative safety, good consumers acceptance and multiple pharmacological activities [44]. The use of vegetal compounds as antimicrobial agents represent an ecological approach, with great therapeutical and preventive value (especially in the management of chronic infections produced by multiresistant and biofilm-growing microorganisms); in some cases, such are the essential oils, their use is limited by their high volatility [46]. In plants, the essential oils seem to play important roles in self-defense against microbial infections and in pollination, as well as in intraspecific communication and as reserve substances [47, 48]. For example, the usnic acid, a secondary lichen metabolite, possess antimicrobial activity against a number of planktonic bacteria, and, as many other secondary lichen metabolites, offers protection to lichen communities against the adherent microorganisms [49, 50]. Our previous results have shown that the usnic acid renders the exposed bacterial cells sensitive to usual doses of antimicrobials, probably acting as a QS inhibitor, which interferes with the coordinate expression of the virulence factors, including the synthesis of adhesins and biofilm development [33]. The usnic acid selectively inhibited biofilm development by Gram positive bacteria and expression of haemolytic properties of the strains isolated from dental plaque, demonstrating its interference with the intra- and inter-species signalling mechanisms based on quorum sensing and response. The growth rate of the isolated strains was changed after contact with usnic acid, by extension of the lag phase to 6-10 h (this time interval being considered as the maintenance time of antimicrobial activity) and by a significant decrease of the viable cell number and prolongation of the generation time [5]. The efficiency of essential oils or polyphenolic extracts obtained from different plants (*Ocimum basilicum, M. Piperita, Mentha sp*. and their synergistic effects as alternative strategies for the treatment of severe infections caused by highly resistant bacteria was tested on: methicillin resistant Staphylococcus aureus (MRSA), extended spectrum beta-lactamases (ESBL) producing *E. coli* and multiresistant *P. aeruginosa* strains. A lot of species have been also investigated by our group for their antimicrobial and anti-infective properties, i.e. *Ribes nigrum, Salvia officinalis* [48], *Eugenia carryophyllata, Citrus maxima, Anethum graveolens, Forsythia vulgare* [51]. A recent article published by Chifiriuc *et al.* (2012) reports the activity of the *Rosmarinus officinalis* and eucalyptol as antibiotic potentiator on *S. aureus* strains isolated from wound secretions and blood cultures [52], converting the tested strains from resistant to oxacilin sensitive, penicillin and to cefoxitin [53]. *Mentha piperita*, commonly called peppermint, a well-known herbal remedy used for a variety of symptoms and diseases [54] has also potentiated the activity of some of the currently used antibiotics (clindamycin, ciprofloxacin, tetracycline, gentamycin, penicillin and erythromycin) in a strain specific manner [55]. In the same study *O. basilicum* essential oils acted also as antibiotic potentiators, with a notable effect on the *S. aureus* oxacillin susceptibility. *E. caryophyllata* essential oil reduced the *Candida albicans* ATCC 10231 biofilm development on the surface of textile dressing sections. Grumezescu and co-workers demonstrated that functionalized magnetite nanostructures could be used as systems for stabilizing the *Eugenia carryophyllata* essential oil on catheter

surface pellicles, in order to prevent or inhibit the fungal biofilm development on the functionalized catheter, highlighting the opportunity of using these nanosystems to obtain improved anti-biofilm coatings for biomedical applications and stabilize these volatile bioactive components [56, 57, 58].

3.4. Propolis and its variaous effects depending on chemical composition

The propolis is a resinous mixture with double origin: vegetal and animal, of vegetable and volatile oils collected by bees from buds and exudates of the plants, which are masticated with bee salivary enzymes and mixed with beewax [59]. One of the major roles of propolis is to protect the bee colony from infectious diseases by its high antiseptic efficacy. Because of multiple chemical components, propolis is considered the most valuable bee product, with a wide variety of therapeutic actions: bactericidal, antiseptic, antiparasitic, antiviral, antitoxic, epithelizant, healing, anti-inflammatory, diuretic, analgesic, antitumoral, regenerating and immunostimulating [60]. Native or prepared as extracts, tinctures and different pharmaceutical forms, the propolis is now one of the most important subjects of study and work for apitherapy [61].

Only relatively a few research works on propolis ability to inhibit biofilm formation have been published. Thus, different teams [62, 63] have shown that propolis inhibits the growth of oral microorganisms and the activity of bacteria-derived glucosyltransferases (GTFs), responsible for glucan synthesis which favors bacterial adhesion and play an essential role in the development of pathogenic dental plaque. Bulman et al. (2011) [59] studied the composition of propolis and revealed that it contains compounds that inhibit signaling mediated by N-acyl-homoserin-lactone in *Pseudomonas aeruginosa PAO1*. The chemical composition of propolis is very complex and varies according to geographical area and plant origin [6]. In propolis there have been identified more than 300 compounds, the major ones being 50-55% resins (pimar acid and abietic acid, benzoic acid, ferulic acid, caffeic acid, cinnamic acid, coumarin and pentacyclic triterpenoids) and balsams, 30% wax and flavonoids, while the minor components are 10% essential oils, 5% pollen, 5% other organic and mineral substances (tannins, enzymes, vitamins etc). The most important fractions responsible for the biological activities of propolis are represented by flavonoids (flavones, flavonols and flavanones) and various phenolic and aromatic compounds [65, 66, 67].

The studies carried out in well known laboratories of pharmacology and medicine have confirmed the important role of this bee product in prevention and treatment of a wide range of diseases. A lot of studies have been carried out to demonstrate the antimicrobial activity of propolis extracts. According to the results of these studies, propolis possesses a broad spectrum against various Gram-positive and Gram-negative bacteria: *Staphylococcus spp., Streptococcus spp., Listeria spp., Bacillus spp., enterobacteria (Klebsiella pneumoniae, Escherichia coli), Pseudomonas aeruginosa, Helicobacter pylori* etc. Some differences concerning the results of the antimicrobial activity testing of propolis extracts are due to: seasonal variation of chemical composition of plants and propolis too, extraction method and various methods for determination of the antimicrobial activity of propolis extracts. The results obtained are also influenced by the experimental conditions (thickness of agar layer, type of agar, inoculum size) and bacterial strains tested [67]. Most studies conducted to determine antimicrobial activity of propolis have shown that propolis exerts a stronger antibacterial action against Gram-positive bacteria, than against Gram negative bacteria. The antibacterial activity of propolis is mainly correlated with caffeic acids, flavonoids and phenolic esters [65, 68]. For this reason, propolis from various geographical areas and with different chemical composition exhibit different activities against Gram-positive and Gram-negative bacteria [65]. Many studies compare the chemical composition of ethanol extracts of propolis and its antimicrobial activity. In 2005, Kosalec showed that those samples of propolis where the flavonoid content was higher than 1% exerted an antimicrobial activity against *S. aureus, S. pyogenes, Enterococcus faecalis, B. subtilis, Candida albicans*. Other studies indicate that the components which are involved in European propolis activity are flavonoids, aromatic acids and esters, while in Brazilian propolis are amyrins (65).

The antibacterial action of propolis was investigated by Grange and Davey [69] using a propolis ethanolic extract (PEE, 70% ethanolic extract) known as "propolis balm"; they applied PEE on bacterial cultures on agar plates, demonstrating its effectiveness in inhibition of the development of *Staphylococcus aureus* strains, including meticillin resistant strains (MRSA).

Our studies have shown that the 30% Romanian propolis tincture presented antibacterial activity towards *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, demonstrated qualitatively and quantitatively by modified agar diffusion and serial two-fold microdilution methods. The antibiofilm activity of propolis tincture, determined by the assay of its inhibitory influence on microbial biofilm formation on inert substratum was recorded only for Gram-positive microbial strain (*S. aureus 13024*) [8]. The results have shown a positive correlation between propolis tincture concentration and decrease of bacterial cell adhesion, similarly with the results of other studies; but other authors [70] reported that PEE inhibited *S. aureus* and *P. aeruginosa* biofilms formation too. Associated with the use of some antibiotics, the efficacy and duration of propolis extract action is more pronounced and these organisms do not develop antibiotics resistance; in the above mentioned study of Helaly and team (2011) demonstrated the antimicrobial and antibiofilm activity of dexapenthanol and propolis extract, alone or in combination with antimicrobial agents on *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains isolated from infected wounds.

4. Conclusion

It can be concluded that a biofilm is a social arrangement, with survival, adaptive and protective value. Biofilm-grown cells are very different from the planktonic, free cells, less efficient in the dissemination and colonization of new environments. The microbial biofilm is considered to be the most successful and competitive expression of the prokaryotic genome - the cells of the biofilm being metabolically more efficient and well protected, exhibiting resistance to different stress factors, including host defence mechanisms and antibiotics. The ability of bacterial cells to exhibit a social behavior is the result of a complex intra- and inter-cellular communication and signaling network, coordinated by a regulation system ubiquitous in bacteria, called *quorum-sensing and response*. This mechanism is implicated in the regulation of very diverse and complex physiological processes, depending on cellular density and mediated by small, self-generated signal molecules known as bacterial pheromones or autoinducers with different chemical structures in Gram-negative and Gram-positive bacteria. The understanding of the mechanism of crosstalk among the bacterial cells, bacterial signal molecules and host cells may contribute to the elaboration of an efficient new strategy for the control of the severity of biofilm associated infections. This new concept is termed „antipathogenic", being not based on the interference with the bacterial growth, but on induction of an under-expression of virulence factors.

Although the potential of vegetal extracts and propolis to be used as therapeutical remedies is known since longtime, their use is still empirical, but the problem of antibiotic resistance as well as the negative impact of the antibiotics discharged in the external environment on the ecological balance has reinforced the studies concerning the characterization of the chemical structures of vegetal products and the active doses, aiming to understand their specific mechanisms of action. The variability of response after the application of these complex natural products (vegetal extracts and propolis) can be a problem, due to their source and to a lot of environmental factors and considering these, it is important to obtain their fractions and assess their specific activities in a dose dependent maner, targets and characterize their spectrum of activity on reference and clinical strains (including resistant and virulent strains), conditions (as for antibiotics) and potential synergistic effects with other substances, because none of the potential or already proved solutions for biofilm associated infections treatment has an absolute value, but only well documented, synergistic combinations can be used.

The anti-infective strategy based on natural products represents a new and ecological approach, with great therapeutical and preventive value in the biomedical field (especially in the management of chronic mono – or polymicrobial infections due to multiresistant and biofilm-forming microorganisms). These products could be also applied in the management of the environment quality, in the agricultural and food and pharmaceutical industries field by reducing the chemical burden delivered in the external medium and by preventing the microbial colonization and biofilm development on different surfaces (pipes and equipments).

The fighting against biofilms associated infections is seen in present as a necessary coordinated strategy based on combinations of antimicrobials, natural QSI, as well as antiinflammatory substances and immunomodulators too, without any selective pressure and disbiosis effect. By this strategy can by avoided the augmentation of antibioresistence phenomenon, maintaining in the same time the eubiosis status of normal microbiota which is so important especially for the habitats intensively populated such as the oral cavity and intestine. For solving the biofilms associated infections there are necessary different methods to assay the antibiotic susceptibility, different therapeutical strategies, medical research on biofilms, clinical trials with the purpose to discover and assess efficient methods/combinations, ecologic alternatives to antibiotics and the QSIs can be a such alternative, with the advantage that these bioactive compounds are not growth inhibitors and selection factors; they are acting only as inhibitors of biofilm formation and/or virulence factors expression by biofilm cells, or disrupting the biofilm' cells cohesion and their communication network, which means rendering them susceptible to some antibiotics in normal therapeutical doses, which is a new concept in the clinical microbiology field.

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