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Antimicrobial Activity of Natural and Synthetic Compounds Against Candida spp., Gram-Positive and Gram-Negative Bacteria

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To my Grandmother
"Nonna Pina"

INDEX

ABSTRACT

Abstract

Antibiotics resistant bacteria have become over the last few decades one of the greatest threats to global health. Infections caused by multi-drug-resistant pathogens are generally difficult to treat and are often fatal. Biofilm formation has been recognized as one of the most important, and most difficult to treat, among the mechanism conferring antibiotics tolerance to pathogens. There is currently an urgent need for novel and effective approaches to fight the global health challenge of antibiotic resistance and in particular biofilm based tolerance. In this context, antimicrobial peptides (AMP) and antimicrobial plant extracts are among the most promising solutions.

This thesis work was aimed at the discovery, characterization and evaluation of the antimicrobial, and especially anti-biofilm, activity of novel AMPs and novel plant extracts. A set of analyses integrating bioinformatics, synthetic biology and biochemical approaches has been employed to identify new antimicrobial compounds. Clinical as well as reference fungal and bacterial strains were used to test the antimicrobial and anti-biofilm potential of these new compounds. Antimicrobial activity assays were performed by microdilution method and time kill assays, while anti-biofilm activity was evaluated with biomass quantification, biofilm vitality assessment and by means of confocal laser scanner microscopy. Finally, cytotoxic experiments were performed against human cells in order to understand if the discovered compounds could be considered promising candidates for the development of topical antimicrobial agents.

As a first step, a cryptic AMP-like peptide named VLL-28, identified in the sequence of an archaeal protein was analyzed and characterized with a good antifungal and anti-biofilm activity against all tested clinical strains.

Then, a second cryptic AMP-like peptide (PAP-A3), derived trough *in silico* screening of human proteins, was identified in the Pepsinogen A activation peptide along with two of its fragments (IMY25 and FLK22). These three peptides exhibited considerable antimicrobial and anti-biofilm properties against a range of pathogenic bacteria, including foodborne organisms infecting the stomach and biofilm producing strains.

Furan-motifs and lignan-motifs were than employed to identify new molecules exhibiting antimicrobial properties. This activity led to the characterization of fourteen synthetic arylfurans and lignan-like arylbenzylfurans, that in turn were tested against Gram-negative

(*Pseudomonas aeruginosa, Escherichia coli*) and Gram-positive bacteria (*Staphylococcus aureus* and *S. epidermidis*). One of these compounds, methyl 4-(2-hydroxybenzyl)-2-phenylfuran-3-carboxylate, was found to be active against *S. aureus* and *S. epidermidis*. This latter showed also a good anti-biofilm activity and was found to be nontoxic against human cells.

Finally, Abietic acid, a tricyclic diterpenoid derived from the resin of pine trees, was tested against *Staphylococcus pseudintermedius* strains and was found to be able to increase the susceptibility to Methicillin in Methicillin-resistant isolates of S. *pseudintermedius* (MRSP) by modulating the expression of *mec* genes. Moreover, Abietic acid also displayed a good anti-biofilm activity by killing almost all cells embedded in biofilm already at very low concentrations.

In conclusion, new possible sources were explored in this work for the creation of novel antimicrobial compounds to fight the emerging antimicrobial resistance to conventional drugs. As a result, a series of new and effective antimicrobial agents have been here detected, characterized and tested against a wide range of dangerous pathogens.

List of Abbreviations

°C	Degree Centigrade
A. baumannii	*Acinetobacter baumannii*
Aa	Amino Acid
AMPs	Antimicrobial Peptides
AS	Absolute Score
ATCC	America Type Culture Collection
B. spizizenii	*Bacillus spizizenii*
C. albicans	*Candida albicans*
CAMPs	Cationic Antimicrobial peptides
Candida spp.	*Candida* species
CD	Circular Dichroism
CDC	Centre for Disease Control and Prevention
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal laser scanning microscopy
CV	Crystal Violet
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ECDC	European Centre for Disease Prevention and
FIC	Fractional Inhibitory Concentration
h	Hour
HCl	Chloridric Acid
HDPs	Host Defense Peptides
HPLC	High Performance Liquid Chromatography
IMAC	Immobilized Metal Ion Affinity Chromatography
K. pneumoniae	*Klebsiellae pneumoniae*
L. monocitogenes	*Listeria monocitogenes*
LB	Luria Bertani Broth
LC–MS	Liquid Chromatography Mass Spectrometry
LPS	Lipopolysaccharides
Maldi-Tof	Matrix-Assisted laser desorption/ionization Time of Flight
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration

MBIC	Minimum Biofilm Inhibitory Concentration
MDR	Multiple Drug Resistant
MIC	Minimum Inhibitory Concentration
Min	Minutes
mL	Milliliter
MOPS	Morpholinepropanesulfonic Acid
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MRSE	Methicillin-Sensitive *Staphylococcus epidermidis*
MRSP	Methicillin-Resistant *Staphylococcus pseudintermedius*
MSSP	Methicillin-Sensitive *Staphylococcus pseudintermedius*
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NB	Nutrient broth
OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
RBCs	Red Blood Cells
RNAase	Ribonuclease
RP-HPLC	High Performance Liquid Chromatography
RPMI	Roswell Park Memorial Institute
S. aureus	*Staphylococcus aureus*
S. enteriditis	*Salmonella enteriditis*
S. epidermidis	*Staphylococcus epidermidis*
S. pseudintermedius	*Staphylococcus pseudintermedius*
S. typhimurium	*Salmonella typhimurium*
SCVs	Small Colony Variant
SD	Standard Deviation
SDA	Sabouraud Dextrose Agar
SDS	Sodium Dodecyl Sulfate
TE buffer	Tris EDTA buffer
TEMED	Tetramethyl Ethylene Diamine Time of Fly
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
WHO	World Health Organization
XTT	2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl) 2H-tetrazolium-5-carboxanilide

µg	Microgram
µl	Microliter
µM	Micromolar

1. INTRODUCTION

Introduction

1.1 Infections

Infectious diseases always constituted a worldwide health issue. Until the beginning of the 20th century, the treatments of many deadly infectious pathologies had been often inadequate. In the years between 1900 to 1980 the discovery of efficacious vaccines and antibiotics marked the beginning of new era characterized by the improvement of sanitation and, subsequently, a marked decline of infection related mortalities [1-3]. The bulk of today's known class of antibiotics was indeed discovered during this period, the so called "golden era of antibiotics drug".
Nevertheless, over the last 20 years we sadly assisted to a resurgence of infection-related fatalities. In fact, in the beginning of the 21th century, infectious diseases are reported to be among the leading causes of death worldwide, despite all of the innovations made in medical research and treatments (Figure 1) [4-7].

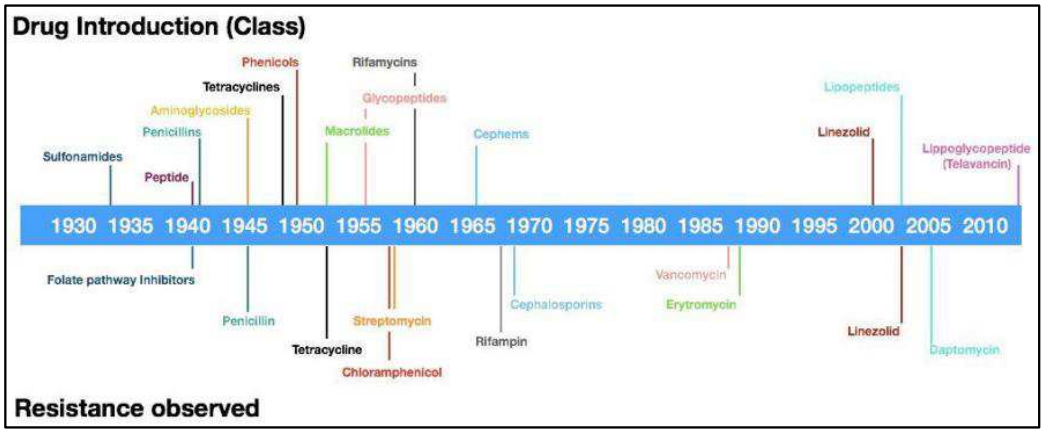

Figure 1. Drug Development and Resistance. Top: *Introduction of major classes of antibiotics.* ***Bottom:*** *First time resistance to the class of antibiotic observed in the clinical setting. Observation of resistance is not equal to loss of clinical efficacy against all clinical isolates. Not all classes or antibiotics are included.*

1.2 Bacterial resistance and tolerance

1.2.1 Antibiotic resistance

According to the World Health Organization (WHO) report, infectious diseases are the third most significant cause of mortality worldwide, with antibacterial drug resistance being reported as one of the most serious issue to address in the control of infectious diseases [8]. The insurgence of drug resistant bacteria has been linked to the artificial selective pressure operated by the widespread and inappropriate use of antibiotics. This phenomenon caused the emergence of multiple-drug-resistant (MDR) bacteria, which especially propagated in hospitals, specialized health centres, and communities.

Compared to other organisms, bacteria are characterized by a fast growth-rate, which makes them able to rapidly modify their genetic background (mainly by means of native genes alteration or horizontal gene transfer) in order to quickly adapt to changing environments. These adaptations eventually lead them to develop new virulence factors and different resistance mechanisms toward currently available antibiotics and antibacterial agents. These mechanisms are mainly represented by: drug target alteration and inactivation, decreased bacterial membrane permeability, that inhibits drugs entering into the cell, overexpression of effluent pumps, that enables drugs expulsion from inside the microorganism, inactivation of antibiotics molecules by means of degradation or modification. A series of bacterial strains, including *Escherichia coli* and *Klebsiella pneumoniae* (beta-lactamase-producers), *Pseudomonas aeruginosa* (carbapenem-resistant), *Staphylococcus spp.* (acquired Methicillin resistant), and *Enterococcus* (vancomycin resistant), have been recognized as the most dangerous among the potentially drug resistant pathogens [9]. Just like antibiotics are used against bacterial infections, antifungal medications are used to treat dangerous fungal infections. And just like some bacterial infections can become resistant to antibiotics, many fungi can no longer respond to the antifungal medications designed to their treatment. This emerging phenomenon, known as antifungal resistance, is usually associated with a high mortality rate and it's a major concern especially for invasive infections of fungi from the genus *Candida*. Among *Candida* isolates, *Candida albicans* represents the predominant species, although in recent years, an increasing incidence of fungal

infections by non-*albicans* species such as *C. parapsilosis, C. tropicalis, C. glabrata* and *C. krusei*, has been observed in hospitals [10]. Although antibiotic-resistant bacterial infections are a widely-recognized public health threat, less is known about the effects of antifungal resistance and the burden of drug-resistant fungal infections [11].

1.2.2 Antibiotic tolerance

In contrast to antimicrobial resistance, drug tolerance is not caused by exposure to antimicrobials, but instead by a phenotypic switch from a metabolically active state, with rapidly-dividing bacteria, to a dormant state, characterized by non-replicating "persister" cells [12]. High levels of antibiotic tolerance have been observed in biofilms where pathogens can live as single cells or alternatively get together. The study of microbial biofilms is relatively recent and emerged only around 4 decades ago with a study by Geesev et. al. [13] Here the authors described for the first time the presence in a natural aquatic system of a slime produced by bacteria themselves which was observed to support micro-colony development and also to anchor bacteria to a specific surface.

1.3 Biofilm

Biofilms are microbial communities characterized by cells attached to a surface and enclosed in a self-produced mucopolysaccharides matrix that not only provides the ability to grow in hostile environments, but also the potential to cause chronic and recurring infections in the host organism [14]. The biofilm matrix consists of extracellular polymeric substances (EPS) such as proteins, including enzymes (<1-2%), DNA (<1%), polysaccharides (1-2%) and RNA (<1%). Water is the mayor contributor of the biofilm volume (up to 97%) and is responsible for the flow of nutrients inside the biofilm matrix [15] (Table 1). This matrix encases the cells within it and facilitates their interaction and communication through biochemical signals as well as gene exchange.

Components	Percentage of matrix
Microbial cells	2-5%
DNA and RNA	<1-2%
Polysaccharides	1-2%
Proteins	<1-2%(including enzyme)
Water	Up to 97%

Table 1. Biofilm chemical composition.

The development of biofilms is an extremely complex process, in which individual cells switch from planktonic to sessile mode of growth. This process comprises four stages: initial adhesion, proliferation, maturation and diffusion (Figure 2).

In the initial adhesion stage, the planktonic cells attach reversibly to a biotic or abiotic surface by means of electrostatic and hydrophobic interactions and microbial surface components, such as fibrinogen or fibronectin. After attaching to a surface, bacteria start dividing and accumulating (proliferation phase). The maturation stage starts when there is an adequate nutrient source and micro-colonies are formed. This is a crucial stage, being characterized by continuous cells proliferation and secretion of polymeric matrices. These latter confer the biofilm a three-dimensional structure composed by mushroom-like cell towers surrounding fluid-filled channels, which support the flow of nutrients through the biofilm. In the final stage, single cells, or larger cell clusters, can be dispersed from the biofilm into their surroundings, thus restarting the planktonic-biofilm cycle. This dispersion is actuated through mechanical forces such as flow in a blood vessel and by detachment factors such as the presence of enzymes that rescind the biofilm matrix. Detachment is a crucial step for the dissemination of bacteria to new niches [16-17].

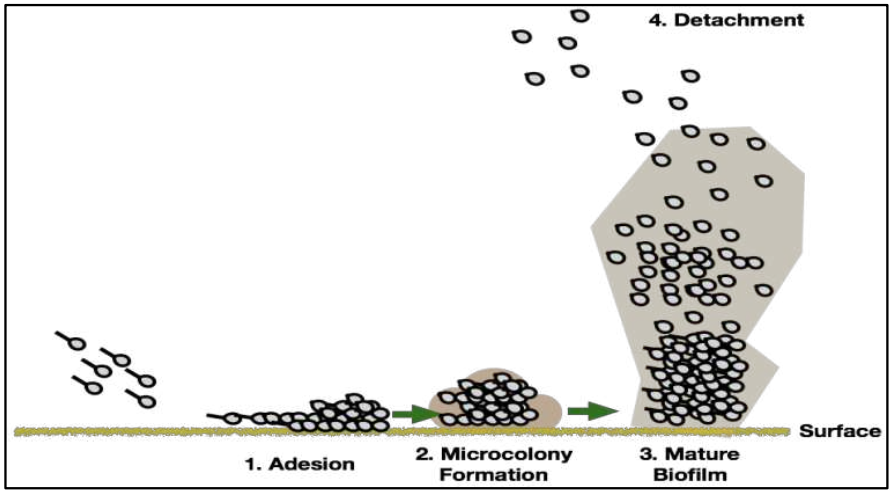

Figure 2. Biofilm formation steps. *1) Adhesion of planktonic cells to the surface; 2) Cells proliferation and micro-colony formation; 3) Biofilm maturation; 4) Dispersion of planktonic cells from the mature biofilm.*

1.3.1 Natural environment and infections

Biofilms are present everywhere in nature and can be formed by single or multiple microbial species. They can be found on rocks and pebbles, on the bottom of the rivers, on the surface of stagnant waters, and in extreme environments that span from extremely hot waters to frozen glaciers. Even if mixed-species biofilms are the most commonly found in nature, single-species biofilms are among the most studied, mainly because they have been found to be involved in a wide variety (by one estimate 80% of the total) of microbial infections in the body [18]. Infectious processes which have been associated with biofilms comprise bacterial vaginosis, urinary tract and catheter infectious, formation of dental plaque, coating contact lenses and less common but more lethal processes such as endocarditis, infection in cystic fibrosis and infections related to medical implants [19] such as joint prostheses, heart valves and intervertebral discs.

1.3.2 Pathogens

Nearly all (99.9%) micro-organisms have the ability to form biofilm on biological or inert surfaces. Biofilm forming ability has been reported in large number of bacterial species such as *Escherichia coli,*

Pseudomonas aeruginosa, Staphylococcus epidermidis and S. aureus [20].

P. aeruginosa is a Gram-negative bacterium found as component of the normal human skin flora [21]. *P. aeruginosa* is a ubiquitous pathogen that can be isolated from diverse sources such as humans, plants and animals. This bacterium is characterized by a strong tendency to form biofilms and these latter have been found to be partially responsible for chronic infections.

S. epidermidis is Gram-positive bacterium with a great potential to cause infections in patients with immune-compromised state, intravenous drug abusers, AIDS affected patients, immuno-suppressive therapy patients and premature new-borns [22]. Moreover, biofilm forming capability has been reported as the main cause of *S. epidermidis* infections during surgical implantations of polymeric devices.

S. aureus a Gram-positive bacterium associated with multi-drug resistance known to be the cause of several nosocomial infections. It grows on catheters and chronic wounds as a biofilm. Some of its cytoplasmic proteins are also acting as matrix proteins providing enhanced flexibility and adaptation to *S. aureus* in forming biofilms during infections.

Over the last decade *S. pseudintermedius* has emerged as a critically important opportunistic animal pathogen. This bacterium is a skin and mucous membrane commensal in the dog and the most frequent pathogen isolated from dogs with dermatologic infections, otitis and wound infections [23]. *S. pseudintermedius* has also implications for public health, as transmission between animals and humans has been described by Paul et al., 2011. An increase in Methicillin-resistant *S. pseudintermedius* (MRSP) strains isolated from infected dogs has been reported worldwide during the last decade [24]. Methicillin resistance is caused by the *mecA* gene, encoding the penicillin-binding protein (PBP) 2a, which shows a low affinity for all β-lactam antimicrobials [25].

1.4 Mechanisms of tolerance in biofilm

The resistant nature of biofilms to antimicrobials and their tendency to grow in implanted medical devices make biofilms a great problem for public health [26]. The traditional mechanisms of antibiotic resistance (drug inactivation, drugs target alteration, reduction of membrane permeability, increase of efflux pumps) are inadequate to

fully explain the strong resistance to antibacterial agents observed in biofilms. Therefore, the existence of some intrinsic mechanisms, responsible for un-conventional antibiotic tolerance, has been suggested in literature [27]. Currently, several mechanisms have been proposed as key factors for the resistant nature of biofilms to drugs [28] (Figure 3).
These mechanisms include [29]:

a) Low penetration of drugs: normally exopolysaccharide matrix can interact with antibiotics by blocking or delaying the access of drugs and/or of the immune system to the inner layers. On the other hand, the matrix can also act as a physical barrier that hinders the penetration of high molecular weight molecules in the biofilm.
   Inactivation of antibiotic drugs takes place during their binding to the biofilm matrix. For example, *P. aeruginosa* biofilm matrix is composed by the anionic alginate exopolysaccharide. The presence of this kind of matrix has been associated with slow penetration of many drugs [30].
b) Neutralization by means of enzymes (such as hydrolases), with drug degrading or inhibiting activity, that are released outside the bacteria.
c) Presence of metabolically inactive and non-dividing 'persister' cells, that pause their replication activity for a limited amount of time in order to guarantee the survival of the community [31]. This phenomenon could weaken the action of all the drugs that are able to attack pathogens only when they are in their growing phase.
d) Several bacterial species have the ability to switch to the small colony variants (SCVs) by developing bacterial subpopulations. These latter are less sensible to the growth phase dependent killing action of some drugs, and have been shown to be resistant to a wide spectrum of adverse environmental conditions. Interaction between genetic elements has been linked to phase variation phenomena [32]. However, differential gene expression alone was not able to fully explain this phenomenon [33].

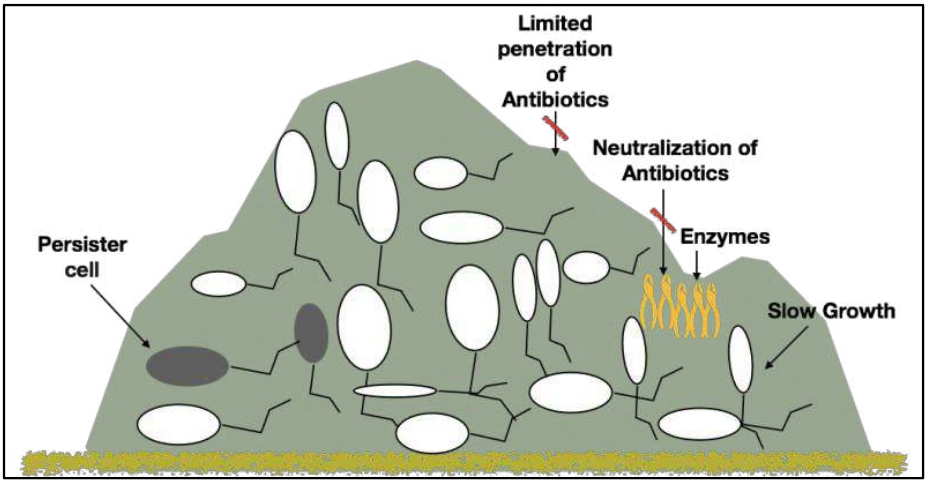

Figure 3. Antibiotic resistance mechanisms associated with biofilm.

1.5 Alternative drugs

Multi-drug Resistance (MDR) represents a global health challenge and is associated with increased morbidity and mortality rates. The annual death-toll is >700.000 people world-wide, predicted to rise to ~10 million by 2050 if current conditions will not change. According to the US Centres for Disease Control and Prevention (CDC), the European Centre for Disease Prevention and Control (ECDC) and the World Health Organization (WHO), we could face a post-antibiotic era in which previously treatable infections will turn to fatal [34]. To mitigate the emerging drug resistance crisis, caused by both bacterial resistance and biofilm tolerance, novel alternatives to conventional antibiotics are to be found by searching for natural sources and/or engineering novel antimicrobial active constructs [35]. Among the many tested compounds, antimicrobial peptides and compounds extracted from plants are gaining increasing attention over recent years. In fact, thanks to their broad spectrum of action against pathogens, the decreased possibility to induce host's resistance and their low toxicity, they are considered among the most promising alternatives to traditional antimicrobial drugs.

1.5.1 Cationic Antimicrobial Peptides (CAMPs)

Antimicrobial peptides (AMPs) are ubiquitously synthetized by almost all living organisms as part of their defence or innate immune system and are of great interest for the development of new antimicrobial compounds [36].
Thanks to their extraordinary properties, such as broad-spectrum activity, rapid action and the difficult development of resistance for microorganisms, antimicrobial peptides are promising molecules to start with in the development of new antibiotics [37]. The number of newly discovered AMPs is continuously growing. Nowadays over 2,000 AMPs, both natural and synthesized, have been documented and submitted to 13 web resources of AMPs present in literature, which give the possibility to get information on them and to conduct peptide analysis [38]. A major group of AMPs is represented by cationic antimicrobial peptides (CAMPs). These peptides are characterized by small linear size (50 aa or less), a positive charge due to basic amino acids such as lysine and arginine, and an amphipathic structure, where a cationic and hydrophobic surface allows the interaction with the biological membrane [36]. Cationic peptides were shown to possess a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria [39]. Unlike eukaryotic cells, bacterial charge surface is anionic. In Gram-negative bacteria, the negative charge of the surface is given by lipopolysaccharides (LPS), which are the major constituents of their outer-membrane. On the other hand, Gram-positive bacteria expose acidic polysaccharides on their surface, such as teichoic and teichuronic acids [40]. By exploiting this feature, peptides could kill bacteria by initially interacting with the extracellular anionic surface, using their positive charge, and then exploiting their amphipathic structure to penetrate the cell membrane, eventually causing the disruption of the membrane integrity and/or the alteration of its electrochemical potential [41]. Membrane disaggregation can either cause a leak of cellular content outside the membrane and/or the alteration of the bilayer composition, leading to reduced membrane functionality and ultimately to cell death [42]. Different models have been suggested in literature to explain the mechanism of CAMP-membrane interaction in bacteria: the aggregate models, the toroidal pore, the carpet and the barrel stave channel [43]. Furthermore, several antibacterial peptides have the ability to penetrate the cytoplasmic membrane and alter the proper functioning of intracellular targets by inhibiting cell wall and/or nucleic acid and

proteins biosynthesis [44]. An example of AMP with such properties is the Buforin II, a DNA-binding CAMP from the Asian toad and PR-39, which has the capability to inhibit at the same time DNA, RNA and protein synthesis [45]. In addition to having a direct antimicrobial function, AMPs also exhibit anti-biofilm activity. For example, lactoferrin has shown good anti-biofilm activity against *S. aureus* and *P. aeruginosa* in medical device infections, especially when administered together with conventional antibiotics, such as rifampicin [46]. In fact, AMPs have the ability to activate regulation of quorum sensing, kill preformed biofilms and inhibit biofilms adhesion and formation [47]. Moreover, most AMPs also display other biological functions such as immunomodulatory properties, promotion of tissue repair through angiogenic roles [48], wound healing [49] and even anti-cancer activity. In fact, AMPs have been recently renamed host defense peptides (HDPs) to account for their immune modulatory functions. As such, well-characterized examples of HDPs are mammals defensins and cathelicidins [50].

Several eukaryotic proteins (e.g. lactoferrin, lysozymes, thrombin, haemoglobin, ribonucleases and histone-like proteins) that are not directly related to host defence mechanisms, can be source of cryptic CAMPs that are released as the product of a partial proteolytic process of these precursor proteins [51]. The iron-binding glycoprotein lactoferrin is a good example of a precursor protein. This protein is ubiquitous in mammalian exocrine secretions. Most of the lactoferrin present in the stomach of infants is proteolytically cleaved by pepsin. As a result, the 25amino acid peptide lactoferricin is released. This latter has been shown to exhibits broader spectrum of antimicrobial activity compared to its precursor [52]. Schmidtchen et al. observed an antibiotic activity for heparin-binding motifs of endogenous proteins [53]. Here the regular spacing of cationic residues in heparin-binding peptides was suggested to be the source of amphipathic/cationic structures that are similar to those exhibited by the vast majority of CAMPs. Interestingly, some CAMPs and cryptic CAMPs exhibit their action not only towards bacteria but also towards eukaryotes (such as fungi), despite the different membrane content and the presence of the cell wall [54]. Regarding this last aspect, less information is currently available on the action of CAMPs on planktonic cells and biofilms of fungal pathogens, such as *Candida*.

1.5.2 Lignan-like compounds

The lignans are a large sub-group of non-flavonoid polyphenols that can be found in more than 70 family of plants. Lignans have several functions in plants, such as antioxidant and anti-inflammatory activity and an active role in the plant's defence against bacteria and fungi infections [55].
These widespread and specialized metabolites are, not only valuable for plants defense machinery, but also important for human health. Epidemiologic studies showed that they can be effective in the prevention of lifestyle-related diseases, such as type II diabetes and cancer. For example, an increased dietary intake of these polyphenols correlates with the reduction in the occurrence of certain types of estrogen-related tumors, such as breast cancer in postmenopausal women. Furthermore, lignans are characterized by a broad range of biological activities in humans, such as anticancer, antioxidant, anti-inflammatory and antimicrobial activity [56].
In fact, plants with elevated lignans content have been adopted as folk medicine in China, Japan, and the Eastern World since ca. 1,000 years [57]. In the last years there has been a renewed interest for this class of compounds, due to the continuous discovery of novel pharmacologically active derivatives, both of natural as well of synthetic origin [58]. In fact, the synthesis and application of multi-substituted furans have gained great interest because of their role as important intermediates in organic synthesis with diverse applications in synthetic, industrial, agricultural, and medicinal chemistry [59].
Despite of the wide structural diversity that exists in the lignans family, the basic lignan backbone consists of two phenyl propanoidic (C6-C3) units, generally linked by a β,β' bond (Figure 4).

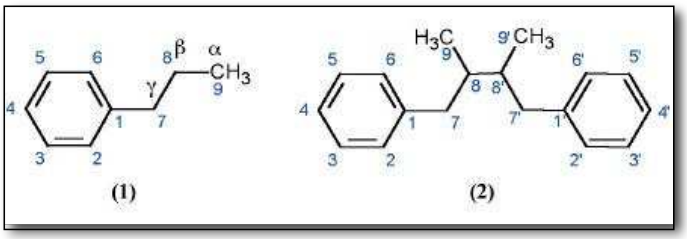

Figure 4. Structure of phenylpropane (1) and lignan (2).

In plants, a wide range of lignans with very different structures can be found. Because of this, lignans are classified in eight subgroups [60-

61]. The main classes of lignans along with their subgroups are reported in Figure 5.

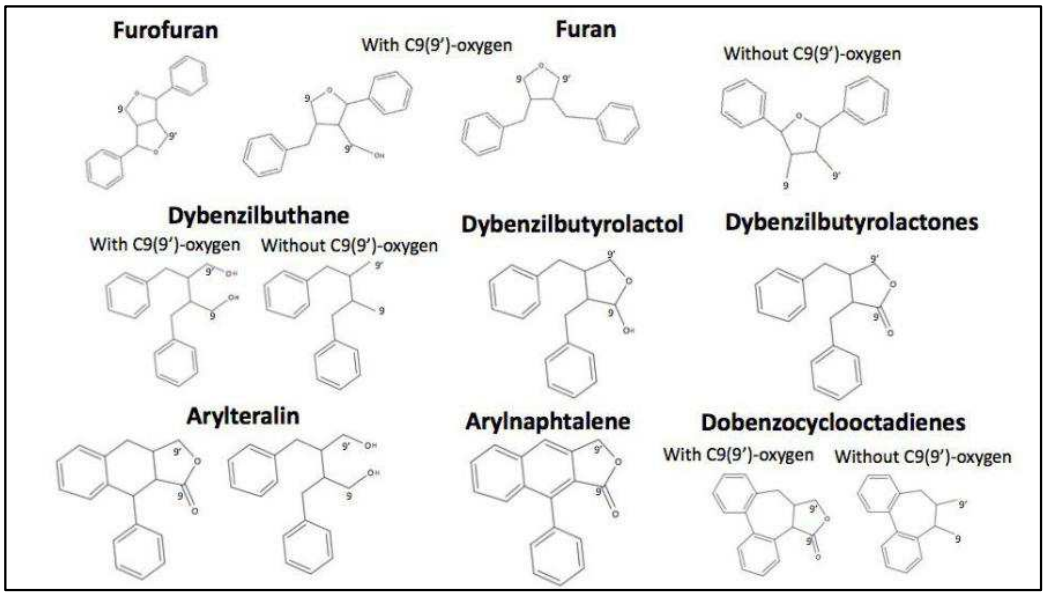

Figure 5. Main sub-class of lignan.

In order to provide an alternative molecular scaffold to lignan compounds isolated from plants, many of them have been modified searching for better analogues showing increased activity, low toxicity or better solubility.

1.5.3 Abietic acid

Abietic acid is the resin of pine trees, but it can be found in many other conifers around the globe. It is a tricyclic diterpenoid that has a well-studied bioactivity profile, including antiviral, antibiotic and antifungal activity [62].
Helfenstein et al. tested the antimicrobial properties of Abietic acid and its derivatives against some of the most common Gram-positive and Gram-negative bacteria. The Authors found that the Abietic acid was able to inhibit by 90% the growth of *S. aureus* and *S. epidermidis* at 63 μgr/mL and 8 μgr/mL respectively, while they observed no effect against *E. coli* and *C. albicans*. Some Gram-negative bacteria, such as *E. coli* and *Pseudomonas spp*., were shown to be able to degrade Abietic acid through P450 enzyme, this latter implied in diterpenoid metabolism [63]. Given these findings, it was not surprising that Abietic acid revealed an inhibitory effect against

Gram-positive bacteria, but not against Gram-negative bacteria. In addition, Abietic acid has been proven not to be toxic for human cells. In fact, Robertson et al. observed no significant reduction in cell viability for up to 100 μM Abietic acid exposure against both human normal skin fibroblast and human metastatic melanoma cell lines [64]. The selective action of Abietic acid on bacterial cells compared to eukaryotic cells, promotes the development of its potential pharmacological applications as alternative antimicrobial agents. Until now, Abietic acid has been extensively tested for almost all common human pathogens. Despite this, there is yet no detailed study addressing the potential antimicrobial properties of Abietic acid against any common animal pathogen. This is an important aspect to study, because in the latest years zoonosis has become an important issue for public health. For example, *Staphylococcus pseudintermedius*, a commensal pathogen of oral cavity and mucocutaneous joint of dog, is one of the most important pathogens responsible of canine pyoderma, especially if carrying resistance genes. *S. pseudintermedius* is not among common human pathogens, but there is a great risk of zoonotic transfer of these organisms and of insurgence of antimicrobial resistance genes from dogs with infections to their owners [65].

2. AIM

Aim of my study

In this PhD project, I focused my attention on the discovery and the study of antimicrobial effects of CAMPs compounds and plant derivatives. In particular, the following studies have been performed.

1) Among cryptic CAMPs, the study of the antifungal and anti-biofilm activity of the VLL-28 peptide, a fragment of the transcription factor Stf76 of *Sulfolobus islandicus* [66], was performed against different *Candida* species.

2) Human zymogens, in particular human aspartic proteases, were processed with a specific bioinformatics tool to extract new putative AMPs. These latter were studied in order to characterize their antimicrobial activity and their mode of action.

3) The antimicrobial potential and anti-biofilm activity of new lignan-like compounds were investigated by using furans and lignans as a source of structural insights.

4) The antimicrobial activity of Abietic acid was studied along with its mechanism of action on bacterial strains with important antibiotic resistant profiles, isolated from animal species.

3. MATERIALS AND METHODS

Materials and Methods

3.1 VLL-28

3.1.1 Strains and culture conditions

Candida strains evaluated in this study included *C. albicans* ATCC10231 reference strain and ten clinical isolates. All clinical strains were obtained from blood cultures of patients admitted to the University Hospital Federico II, Naples. Identification was performed by sub-culturing on chromogenic agar (Chromid agar) (Becton Dickinson) or by biochemical characterisation using the Vitek II system (Biomerieux) and confirmed by MS Maldi-Tof (Bruker). Susceptibility to Amphotericin B, Anidulafungin, Micafungin, Caspofungin, 5-Fluorocytosine, Posaconazole, Voriconazole, Itraconazole, Fluconazole was performed by using Sensititre Yeast One, colorimetric microdilution method (Termofisher). All strains were stored as a 15% glycerol stock at -80°C. Prior to each experiment, cells were sub-cultured from stock onto Sabouraud dextrose agar (SDA) (Becton Dickinson) at 37 °C for 48h.

3.1.2 Compounds

VLL-28 peptide (VLLVTLTRLHQRGVIYRKWRHFSGRKYR) and its fluoresceinated derived form FITC-VLL-28, named VLL-28* (VLLVTLTRLHQRGVIYRKWRHFSGRKYRGK*), bearing the chromophore fluorescein coupled to the last lysine residue at the C-terminus, were synthetized and purified to 95% homogeneity by Inbios (Napoli, Italy) as assessed by LC–MS.

3.1.3 Determination of the minimum inhibitory concentration (MIC)

Antifungal activity of VLL-28 peptide was determined according to a standardized broth microdilution method [Clinical and Laboratory Standards Institute (CLSI) document M27-A2]. Briefly, for each isolate, the cell suspension, after overnight culture, was adjusted to $3x10^3$ CFU/mL in RPMI 1640 medium supplemented with 0.2% glucose and buffered with morpholinepropanesulfonic acid (MOPS)

and 100 µL aliquots were added into the 96-well microtiter plate. Peptide stock solution was serially diluted in RPMI medium and 100 µL aliquots were transferred in microplate, giving final concentrations ranging from 0,09 µM to 100 µM before incubation for 48 h at 37 °C. Amphotericin B was used as control. The MIC was taken as the lowest concentration of the peptide resulting in complete inhibition of visible growth after 48 h incubation. The concentration values were tested three times in each independent experiment. Minimum Fungicidal Concentration (MFC) was determined by transferring 50 µl aliquots of each sample treated with concentrations equal or higher than the MIC onto SDA plates, and incubating plates at 37°C for 24 h. The highest dilution that yielded no fungal growth on agar plates was taken as MFC.

3.1.4 Intracellular localization assay

Confocal laser scanning fluorescence microscopy (CLSM) was used to study the intracellular target of the peptide. Double staining of *C. albicans* and *C. tropicalis* strains with FITC-labelled peptide and MitoTracker Orange (chloromethyl-H_2-tetramethyl rosamine, Molecular Probes), a permanent mitochondrion-selective dye, was achieved as follows. A *Candida* cell suspension (200 µl; $3.2x10^6$ cells/mL of PBS) was incubated with 150 nM MitoTracker Orange for 15 min at 37 °C. Cells were washed with 200 µl of PBS and treated with FITC-labelled peptides at 25 µM and 12.5 µM, for *C. albicans* and *C. tropicalis* respectively, for 15 min and 2 h. Cells were collected by centrifugation (5 min at 10000 g), suspended in 20 µL of PBS and examined by confocal microscopy with confocal laser-scanning microscope (Zeiss) equipped with immersion oil 63X objective lens.

3.1.5 In vitro biofilm formation assay

Biofilms of tested *Candida* strains were formed in flat-bottomed 96-well microplates as described by Stepanovic with modifications [67]. Firstly, for each strain, a cell suspension in opportune medium supplemented with 2% glucose for *Candida* spp. was adjusted to $1x10^5$ CFU/mL. Next, plate wells were inoculated with 200 µL of standardised yeast and bacterial suspension in triplicate and incubated at 37 °C for 24 h to allow biofilm formation. Then, the culture broth

in excess was aspirated gently, and each well was washed twice with PBS and dried at 60 °C for 30 min. Biofilm formation was observed upon staining with 50 µL of 1% crystal violet for 30 min. The wells were again rinsed to discard crystal violet; each well was washed with PBS and then 150 µL of absolute ethanol was added to release the colorant into the medium. Absorbance was measured at 595nm using a Biophotometer (Eppendorf) to assess the amount of biofilm produced. We used the classification introduced by [67] with modifications. The tested isolates were classified into four categories: non-adherent (NA), weakly (WA), moderately (MA), or strongly (SA) adherent.

3.1.6 Candida spp. adhesion inhibition assay

The adhesion of *Candida* spp. strains was assayed using flat-bottomed 96-well microplates. For each isolate, 100 µl of cell suspension in RPMI medium adjusted to $1x10^6$ CFU/mL was incubated with 100 µl of RPMI containing serially double-diluted peptide concentrations in order to obtain final sub-MIC concentrations for each strain, ranging from 3 to 50 µM. The plates were then incubated at 37 °C with shaking (100 rpm). The positive control included peptide free wells. After 90 min incubation for the adhesion phase, the medium with unbound peptide was aspirated, non-adherent cells were removed by washing the wells with PBS, and 200 µL of fresh RPMI was added. The plate was further incubated at 37 °C for 24 h and after the incubation period, a crystal violet assay was performed as described below. Adhesion inhibition activity of the peptide was expressed as the minimum biofilm-inhibiting concentration (MBIC), which is defined as the minimum concentration of the peptide resulting in 80% reduction of the biofilm biomass formation compared to the growth control sample.

3.1.7 Effect of VLL-28 against preformed biofilm

To assess the activity of the compound against mature biofilm, *Candida* biofilms were allowed to form in each well of 96-well microtiter plates, as described above. After 24 h planktonic cells were removed from the wells and the plates were rinsed with 200 µL of PBS. Cells adhered to the microtiter plates were exposed to 200 µL of compounds diluted in broth at the concentration from 6 µM to 100

μM. The plates were further incubated for 24 h at 37 °C. Non-treated cells were incubated with 200 μL of RPMI broth, which served as a growth controls and wells incubated with only medium as sterile controls. At the end of the experiment crystal violet-staining was performed to assess biofilm biomass.

3.1.8 Quantitation of metabolic activity of mature biofilm by XTT assay

To quantify the metabolic activity of mature biofilms of *Candida* strains, the XTT [2,3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Roche Diagnostics) reduction assay was used. After compounds treatment, the planktonic cells were removed and the wells were washed twice with PBS. Successively, 150 μL of XTT was added to each well and the plate incubated in the dark for 40 min at 37 °C. The reduction of the tetrazolium salt by dehydrogenase, enzymes of metabolically active cells in biofilms, into orange formazan dye that could be photometrically measured at 490 nm. The change of the absorbance of XTT was employed for studying the drug effect on bacterial biofilm. The XTT alone was set as negative control. Viability values were compared for each well with respect to controls. Anti-biofilm activity of the peptide was expressed as the minimum biofilm eradicating concentration (MBEC), which is defined as the minimum concentration of the peptide resulting in 80% disruption of the biofilm compared to the growth control.

3.1.9 Confocal laser scanning microscopy

CLSM was used to illustrate the effect of peptide on viability and architecture of mature biofilms of *Candida* strains. *Candida* cells were grown on Nunc® Lab-Tek® II chambered cover-glasses (Sigma) suited to confocal microscopy applications, in a static condition for 24 h. VLL-28 was added on a 1-day-old biofilm at 2xMIC value for each strain. *Candida* suspensions without compound was used as a positive control. After 24 h, biofilms were rinsed with PBS and stained by using a LIVE/DEAD® FungaLight Yeast Fungal Viability stains (Life Technologies, Monza, Italy). Briefly 200 μL of the water solution containing SYTO9 and propidium iodide (PI), mixed in a ratio of 1:1, were added to the well

and were incubated at room temperature for 15 min in the dark. After incubation, stain was removed and biofilm was washed with water. The images were observed using a LSM 700 inverted confocal laser-scanning microscope (Zeiss, Arese, Milano, Italy). The whole wells were observed to see the biofilm formation and its global structure. Three different areas of each well were scanned using a 10X objective lens with the signal recorded in the green channel for Syto9 (excitation 488nm, emission 500–525nm) and in red channel for PI (excitation 500-550nm, emission 610-650nm), with pinhole open to 1. Cells with intact cell membranes showed a green fluorescence, whereas those with damaged cell membranes were red.

3.1.10 Statistical analysis

All experiments were performed in triplicate, the results are expressed as mean ± standard deviation (SD). Student's t test was used to determine statistical differences between the means, and $p<0.05$ was considered a significant difference.

3.2 Pepsinogen A3 derived peptides

3.2.1 Strains and culture conditions

Strains included *Escherichia coli* ATCC25922, *Enterococcus faecalis* ATCC29212, *Acinetobatcer baumanii* ATCC17878, *Klebsiella pneumoniae* ATCC700603, *Salmonella enterica serovar typhimurium* ATCC14028, *S. enteriditis* 706RIVM, *Bacillus subtilis subp. spizizenii* ATCC6633, *Pseudomonas aeruginosa* ATCC27853, *P. aeruginosa* PAO1, *P. aeruginosa*AA2*, P. aeruginosa*KK27 and *Staphylococcus aureus* ATCC6538P. *S. aureus* MRSA and *Listeria monocytogenes* clinical isolates admitted to the University Hospital Federico II, Naples. All strains were stored as a 20% glycerol stock at -80°C. For the activation, all strains were cultivated into Nutrient broth (Becton Dickinson) at 37 °C for 24 h.

3.2.2 Recombinant production of pepsinogen A3 derived peptides

PAP-A3 and its derived peptides were produced in *E. coli* as fusion proteins with a new carrier derived from modified onconase from *Rana pipiens* by Prof. Notomista's group of the Department of Biology, University of Naples Federico II. Recombinant proteins were expressed in *E. coli* BL21 (DE3) (Novagen, San Diego, CA, USA) and purified from inclusion bodies as previously described for ONC-DCless-H6-(P)GKY20 fusion protein [68]. Recombinant proteins, purified by IMAC (Ni Sepharose^{TM} 6 Fast Flow was from GE Healthcare, Uppsala, Sweden), were cleaved in mild acid conditions (incubation for 24 h at 60°C in 0.1 M acetic acid, 18 mM HCl, pH 2.0, under nitrogen atmosphere). Peptides were purified by carrier selective precipitation at neutral pH. Cleavage efficiency of the DP sequences into the linker region was determined by densitometric analysis of 20% SDS-PAGE (ChemiDoc detection system, Bio-Rad; Quantity One software). Degree of fragmentation at internal cleavage sites was calculated by normalization of the RP-HPLC peak areas of peptides mixtures at neutral pH, with extinction coefficient calculated by the ProtParam tool. The identity of peptides was assessed by mass spectrometry analyses. Details are reported into supporting information. Purified peptides were lyophilized and stored at -80°C.

3.2.3 Determination of the minimum inhibitory concentration

The MIC values of human pepsinogen A3 isoform derived peptides against planktonic bacteria were examined by broth microdilution method previously described for antimicrobial peptides [69] with minor modifications. All peptides were dissolved in sterile water (cat. P5950, Romil PUROM, ROMIL Ltd Cambridge), and dispensed into aliquots stored at -20°C until use. Twofold serial dilutions of peptides were carried out in sterile 96-well polypropylene microtiter plates (cat. 3879, Costar Corp., Cambridge, MA) at concentrations ranging from 0.09 to 50 μM. Bacteria from an overnight culture were inoculated in Nutrient broth medium to a final concentration of 2-5x10^5 CFU/mL per well., polymyxin B and vancomycin were used as control. Plates were incubated at 37°C for 24 h. The MIC values were determined as the lowest concentrations of different compounds at which no visible bacterial growth was observed.

3.2.4 Time-kill assay

Bactericidal activity was tested by a plate viable-count method performed as previously described [70] with minor modifications. Briefly, overnight cultures were diluted in Nutrient Broth 0.5X (Becton-Dickenson, Franklin Lakes, NJ) to give final concentrations of ~ $5x10^6$ CFU/mL (200 µL). Bactericidal activity of (P)PAP-A3 was tested at 10 µM final concentration. Assays were carried out at pH 7.0 and 3.5 (acidified by diluted HCl addition). Cell viability was also tested by control curves in the absence of peptide to evaluate any effects of pH on the growth. Samples were incubated at 37 °C for 45 min, and 10 µL of each mixture was then taken at 0, 15, 30 and 45 min and neutralized with 990 µL of Nutrient Broth 0.5 X containing 5 mM sodium hydrogen carbonate. Bacterial suspensions (100 µL) were plated on LB agar plates, and viable counts were determined after incubation at 37 °C for 18 h. Experiments were carried out twice independently for each bacterial strain.

3.2.5 Determination of the minimum inhibitory concentration and minimal bactericidal concentration in like-biofilm-condition

The experiment previously described (section 3.1.3) was repeated for *S. aureus* ATCC6538P and *P. aeruginosa* PAO1 strains in the biofilm conditions: glucose added (0,5%) to medium (NB 1%). The minimal bactericidal concentration (MBC) of all peptides was determined by sub-culturing the dilutions from each well without visible bacterial growth on Trypticase Soy Agar (TSA) at 37 °C for 24 h. The first peptides dilution plate with no visible bacterial growth after incubation was determined as MBC.

3.2.6 Effect of peptides on microbial biofilm formation

One hundred µl of the tested peptides at the concentrations ranging from 0,19 µM to 6,25 µM was added to 100 µL broth containing 0.5% glucose and at a final density of 10^6 CFU/mL for *S. aureus* ATCC6538P and *P. aeruginosa* PAO1. Non-treated cells were incubated with 200 µL of opportune medium broth, which served as a growth control. After culturing for 24 h at 37 °C, the supernatant was removed completely, and the wells were rinsed twice with PBS. The amount of biofilm formed in the wells was measured by staining with

0,1% CV and the absorbance of the solution was measured at 595 nm. The percentage of biofilm mass reduction was calculated using the following formula: [(Ac−At)/Ac] × 100, were Ac is the OD_{595} for control well and At is OD_{595} for treated biofilm.

3.2.7 Effect of PAP3A, (P)IMY25 and (P)FLK22 against preformed biofilm

The method used to assess the activity of the peptides against *S. aureus* and *P. aeruginosa* mature biofilm is described in the section 3.1.7 with few modifications. Biofilms were allowed to form in each well of a polypropilene 96-well microtiter plate. Peptides were added on a 1-day-old biofilm at the concentration ranging from 6,26 μM to 25 μM.

3.2.8 Quantitation of metabolic activity of mature biofilm by XTT assay

See section 3.1.8

3.2.9 Biofilm cultivation in static chambers and confocal microscopy

S. aureus ATCC6538P and *P. aeruginosa* PAO1 were grown in the chambered cover glass (μ Slide 4 well; Ibidi GmbH, Munich, Germany), suited to confocal microscopy applications, in a static condition for 48 h. The assay is the same described in the section 3.1.9.

3.2.10 Biofilm cultivation in flow chambers and confocal microscopy

Biofilms were grown for 72 h in the absence or presence of each PAP-A3 derived peptide (3 μM) at 37°C in flow chambers with channel dimensions of 1 by 4 by 40 mm. The medium used was BM2 minimal medium [62 mM potassium phosphate buffer, pH 7.0, 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 μM $FeSO_4$] containing 0.4 % (w/v) glucose as a carbon source. Silicone tubing (inner diameter, 0.062 in.;

outer diameter, 0.125 in.; wall thickness, 0.032-in.; VWR) was used, and the device was assembled and sterilized by pumping a hypochlorite solution through the system for 5 min using a multichannel peristaltic pump. The system was then rinsed with sterile water and medium was introduced into each channel. Flow chambers were inoculated by injecting 400 µL of a *P. aeruginosa* PAO1 wild type. Chambers were incubated overnight and cultures were diluted to an OD at 600nm of approximately 0.05. After inoculation, chambers were left without flow for 3 h, after which medium was pumped through the system at a constant rate. Bacteria were allowed to develop structured 2-day-old biofilms prior to peptide treatment for the following 8 h. Biofilm cells were stained using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR) using a 1:1 ratio of SYTO 9 (green fluorescence, all cells) to propidium iodide (PI; red fluorescence, dead cells). Microscopy was carried out using a confocal laser scanning microscope (Zeiss LSM 700 Laser Scanning Confocal), and three-dimensional reconstructions were generated using the Imaris software package (Bitplane AG). Experiments were performed twice with identical results.

3.2.11 Circular dichroism

CD measurements were performed by Prof. Notomista's group and were recorded on a Jasco-720 spectropolarimeter over the wavelength interval of 190-260nm, at 20 °C using a 0.1 cm optical path length quartz cuvette. Peptides were dissolved in 10 mM sodium phosphate buffer (pH 7.0) at concentrations of 50 µM [(P)IMY25 and (P)FLK22] and 25 µM for (P)PAP-A3. CD spectra were recorded with a time constant of 4 s, a 2nm bandwidth, and a scan rate of 10nm min^{-1}. The spectra were signal-averaged over at least three scans and the baseline was corrected by subtracting the complete buffer spectrum. Spectra were recorded in the presence of increasing concentrations of seaweed alginate (Sigma Aldrich): 0.02-0.04-0.08-0.16-0.32 mg/mL. In this case, the baseline was corrected by subtracting the spectrum of alginate alone at the same concentration tested.

3.2.12 Haemolytic assay

EDTA anti-coagulated mouse blood was centrifuged for 10 min at 800×g (20 °C) to sediment the red blood cells. Pelleted RBCs were washed three times and diluted 200-fold in PBS. In 96-well polypropylene plates, 75 µL of two-fold serially diluted peptides (from 40 up to 0.31 µM) were mixed with an equal volume of RBC suspension and incubated for 1 h at 37 °C. PBS was used as negative control (blank) whereas 0.2% (v/v) Triton X-100 solution as positive control. Supernatants, collected after 10 min centrifugation at 1300×g (20 °C), were transferred into polystyrene 96-wells plates and absorbance was measured at 405 nm. Hemolysis (%) was calculated as follows: $[(A_{Peptide} - A_{Blank}) / (A_{Triton} - A_{Blank})] \times 100$.

3.2.13 Cytotoxicity on mammalian cells

Cells surviving upon peptide treatment was assessed on HaCaT cells by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay. HaCaT cells ($1x10^4$ cells/well) were grown for 24, 48 and 72 h in absence or presence of increasing concentrations (5- 10- 20- 40 µM) of peptides (P)PAP-A3, (P)IMY25 and (P)FLK22. Cell survival values were expressed as the percentage of viable cells with respect to control untreated samples. Cytotoxicity experiments were performed three times independently.

3.2.14 Statistical analysis

See section 3.1.10.

3.3 Lignan-like compound

3.3.1 Strains and culture conditions

The Gram-negative strains: *P. aeruginosa* ATCC27853 and *E. coli* ATCC25922 and the Gram-positive strains: *S. aureus* ATCC12600 and *S. epidermidis* ATCC35984 were obtained from the American Type Culture Collection (Rockville, MD). Clinical strains of *S.*

aureus (MRSA), *S. epidermidis* (MRSE) were obtained from specimens of patients admitted to the University Hospital Federico II, Naples. Identification was performed by sub-culturing on Tryptic Soy Agar (TSA, Becton Dickinson) and MacConkey (Biomerieux) and by biochemical characterisation using the Vitek II system (Biomerieux) and confirmed by MS Maldi-Tof (Bruker). All strains were stored as a 20% glycerol stock at -80 °C. Before each experiment *P. aeruginosa, E. coli* and *S. epidermidis* were cultured in Luria-Bertani (LB, OXOID) broth under aerobic conditions at 37 °C for 24 h on an orbital shaker at 120 rpm. *S. aureus* was cultured in tryptic soya broth (TSB, OXOID) under aerobic conditions at 37 °C, 200 rpm for 24 h.

3.3.2 Preparation of furan derivatives

Arylfurans 1-3, arylbenzylfurans 4-11 and new derivatives 12-14 were synthesized by Prof. Della Greca's group of the Department of Chemical Sciences, University of Naples Federico II. All used reagents (triphenylphosphine oxide (Ph_3PO), trifluoromethylsulfonic anhydride (Tf_2O), phenol) and solvents were obtained from commercial suppliers (Sigma-Aldrich). Trifluoromethylsulfonic anhydride (Tf_2O) (0.1 mL, 0.6 mmol) was added at 0° to a solution of triphenylphosphine oxide (Ph_3PO) (0.6 mmol) in dry CH_2Cl_2 (1 mL), and the mixture was stirred for 15 min at r.t. Then, phenol (0.5 mmol) and furan 2 (0.5 mmol in 1 mL of dry CH_2Cl_2) were added and the mixture was stirred. Upon completion of the reaction (1 h), the organic solvent was evaporated and the residue was obtained by preparative silica gel thin-layer chromatography (TLC) using ethyl acetate-CH_2Cl_2, 10% as eluent, to give pure 12 (28%): IR (CH_2Cl_2): 3201, 1720, 1579, 1486, 1324, 1081 cm^{-1}. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.63 (d, 2H, J=9.0 Hz, H-2' and H-6'), 7.21 (s, 1H, H-5), 7.19 (dd, 1H, J= 7.8, 1.5 Hz, H-6"), 7.14 (m, 1H, H- 4"), 6.93 (d, 2H, J=9.0 Hz, H-3' and H-5'), 6.90 – 6.86 (m, 2H, H-3" and H-5"), 3.98 (s-, 2H, CH_2), 3.85 (4′MeO), 3.79 (MeO). ^{13}C-NMR (101 MHz; $CDCl_3$): δ 165.9 (COOMe); 160.3 (C-4'); 158.8 (C-2"); 153.9 (C-2); 139.9 (C-5); 130.4 (C-6"); 130.1 (C-2' and C-6'); 128.0 (C-4"); 126.1 (C-1'); 125.6 (C-1"); 120.6 (C-5"); 122.8 (C-3); 116.5 (C-3"); 113.4 (C-3' and C-5'); 111.3 (C-4); 55.2 (MeO); 51.6 (4′MeO); 24.9 (-CH_2-). EI-MS: m/z = 338.11 $[M]^+$. The above procedure using furan 3 and phenol gave a mixture of 13 and 14 that was further subjected to preparative TLC (ethyl acetate- CH_2Cl_2, 10% as eluent)

to give pure 13 (39%) and 14 (44%).
13: IR (CH_2Cl_2): 3202, 1719, 1639, 1497, 1290, 1084 cm^{-1}. ^1H -NMR (400 MHz, $CDCl_3$): δ 7.59 (d, 2 H, J = 6.6 Hz, H-2' and H-6'); 7.57 (d, 2H, J = 6.6 Hz, H-3' and H-5'); 7.25 (s, 1 H, H-5); 7.21 (dd, 1H, J = 7.6, 1.1 Hz, H-6"); 7.15 (td, 1 H, J=7.7, 1.6 Hz, H-4"); 6.95-6.85 (m, 2 H, H-3" and H-5"); 4.00 (s, 2H, - CH_2-), 3.82 (s, 3H, MeO). ^{13}C -NMR (101MHz; $CDCl_3$): δ 165.5 (COOMe); 157.2 (C-2); 153.7 (C-2"); 140.8 (C-5); 131.3 (C-3' and C-5'); 130.4 (C-6"); 130.1 (C- 2' and C-6'); 129.1 (C-1'); 128.0 (C4' and C-4"); 126.1 (C-1"); 125.9 (C-3); 121.7 (C-5"); 116.4 (C-3"); 112.9 (C-4); 51.9 (MeO); 24.9 (-CH_2-). EI-MS: m/z = 386.2 $[M]^+$.
14: IR (CH_2Cl_2): 3201, 1718, 1639, 1495, 1300, 1084 cm. H-NMR (400MHz, $CDCl_3$): δ 7.66 (d, 2 H, J=10.5 Hz, H-2' and H-6'); 7.56 (d, 2H, J = 10.5 Hz, H-3' and H5'); 7.13 (d, 2 H, J = 8.1 Hz, H-2" and H-6"); 7.06 (s, 1 H, H-5); 6.80 (d, 2H, J = 8.1 Hz, H-3" and H-5"); 3.92 (s, 2 H, -CH_2-), 3.75 (s, 3H, MeO). ^{13}C-NMR (101 MHz; $CDCl_3$): δ 164.2 (COOMe); 157.2 (C-4"); 154.0 (C-2); 140.3 (C-5); 132.0 (C-1'); 131.7 (C-2' and C-6'); 129.9 (C-3' and C-5'); 129.8 (C-2" and C- 6"); 128.0 (C-1"); 127.5 (C-3 and C-4'); 113.7 (C-4, C-3" and C-5"); 51.3 (MeO); 30.5 (-CH_2-). EI- MS: m/z = 386.02 $[M]^+$.

3.3.3 Determination of the minimum inhibitory concentration

MIC of the tested compounds were determined in LB and TSB medium by the broth micro-dilution assay, following recommendations of CLSI. Bacterial suspensions were diluted to yield an optical density (OD) around 0.5 at 595nm (about 1.4×10^7 CFU/mL for *S. aureus* and 4.5×10^8 CFU/mL for *S. epidermidis*), and then further diluted to a final concentration of 1×10^6 CFU/mL. One hundred μL of the compound under investigation were added to 100 μL of bacterial cell suspension in each well of the microplate to yield a final cell concentration of 5×10^5 CFU/mL and a final compounds concentration from 0.25 μg/mL to 128 μg/mL. A few wells in each plate either did not have any bacteria, in order to test the sterility of the medium, or did not have any compound, in order to check for bacterial viability. Negative control wells were set to contain bacteria in LB or TSB broth plus the amount of DMSO used to dilute each compound. Positive controls included tobramycin and vancomycin (ranging from 0.25 μg/ml to 4 μg/ml). After an incubation of 24 h at 37 °C medium turbidity was measured by a microtiter plate reader (Tecan, Milan, Italy) at 595nm. Absorbance was proportional to

bacterial growth.

3.3.4 Cytotoxicity on eukaryotic cells (MTT assay)

The cytotoxicity assay was performed on human immortalized keratinocytes cells (HaCaT), cultured in DMEM supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Semi-confluent HaCaT cells (10^3/well), treated or not with various concentrations (ranging from 2 µg/mL to 128 µg/mL) of Compound 11 and 13, were grown in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl DMEM, at 37 °C and 5% CO_2. After 8, 24 and 48 h 10 µl of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) labelling reagent (Roche Diagnostics, Basel, Switzerland; final concentration 0.5 mg/mL) were added to each well. Four hours after the incubation time, 100 µl of the solubilisation solution (10% SDS in 0.01 M HCl) were added and the cultures were incubated overnight. The spectrophotometric absorbance was measured using a microplate ELISA reader (Biorad) at 570nm wavelength. HaCaT cells incubated with DMSO-PBS served as an untreated control.

3.3.5 Determination of rate of kill

Assays for the killing rate of *S. aureus* and *S. epidermidis* by the Compound 11 were carried out as previously reported with slight modification [71]. 100 µL of inoculums density, approximately 10^5 CFU/mL, were added to microplates with 100 µl of Compound 11 at the concentration of 4 µg/mL. The plates were incubated at 37 °C on an orbital shaker at 120 rpm. Viability counts were performed at 0, 2, 4, 6, and 24 h of incubation at 37 °C by plating 0.1 mL undiluted and 10-fold serial diluted samples onto LB plates in triplicate. After incubating at 37 °C for 24 h, emergent bacterial colonies were counted, CFU/mL calculated, and compared with the count of the culture control.

3.3.6 In vitro biofilm formation assay

To evaluate the ability to form biofilm of tested strains was used the assay described into the section 3.1.5 with the modifications about the amount of percentage of glucose used. The cell suspensions in medium were supplemented with 0.5% glucose.

3.3.7 Effect of Compound 11 on biofilm formation

The method used to assess the activity of the peptides against biofilm formation described in the section 3.2.6.
Compound 11 was added at sub-MIC concentrations ranging from 0.5 μg/mL to 2 μg/mL.

3.3.8 Effect of compounds against preformed biofilm

The method used to assess the activity of the peptides against mature biofilm is described in the section 3.1.7.
Compound 11 was added to the wells at the concentrations ranging from 8 μg/mL to 32 μg/mL.

3.3.9 Quantitation of metabolic activity of mature biofilm by XTT assay

See section 3.1.8.

3.3.10 Confocal laser scanning microscopy

S. aureus and *S. epidermidis* 1-day-old-biofilm were treated with the Compound 11 at the concentration of 32 μg/mL. The assay is the same described in the section 3.1.9.

3.3.11 Statistical analysis

See section 3.1.10.

3.4 Abietic acid

3.4.1 Bacterial strains

Methicillin sensitive and resistance *Staphylococcus pseudintermedius* strains, MSSP and MRSP respectively, were obtained from clinical sample of dogs from the Microbiology Laboratory of the Department of Veterinary and Animal Production, University of Naples "Federico II".

3.4.2 Compound

Abietic acid was dissolved in pure ethanol (99%) before the use.

3.4.3 Determination of the minimum inhibitory concentration and minimal bactericidal concentration

The antibacterial activity of Abietic acid was determined by the assay described in the section 3.2.3.
Abietic acid was tested with concentration ranging between 0,06 μg/mL to 128 μg/mL. Negative control wells were set to contain bacteria in LB or TSB plus the amount of DMSO used to dilute each compound. Positive controls included tobramycin and vancomycin (ranging from 0.25 μg/ml to 4 μg/ml).
Minimum bactericidal concentration (MBC) was defined as the concentration that caused a 3-log viability decrease in the initial inoculum and was calculated by colony counts on TSA plates incubated for 24 h at 37 °C.

3.4.4 Determination of rate of kill

The method used to assess the activity of the peptides against biofilm formation is described in section 3.3.5.
Abietic acid was added at the concentrations of 32 μg/mL.

3.4.5 Checkerboard assay

The interaction between Abietic acid and Methicillin against MRSP was evaluated by the checkerboard method in 96-well microtiter plates containing TSB. Briefly, Abietic acid and Methicillin were serially diluted along the y and x axes, respectively. The final antimicrobial substance concentrations (after two-fold dilution) ranged from 1/16 to 1-fold the MIC for Methicillin and from 1/64 to 2-fold the MIC for Abietic acid. The checkerboard plates were inoculated with bacteria at an approximate concentration of 10^5 CFU/mL and incubated at 37 °C for 24 h. Next, bacterial growth was assessed visually and the turbidity measured by microplate reader at 595nm. To evaluate the effect of the combined treatment, the fractional inhibitory concentration (FIC) index for each combination was calculated as follows: FIC index = FIC of Abietic acid + FIC of Methicillin, where FIC of Abietic acid (or Methicillin) was defined as the ratio of MIC of Abietic acid (or Methicillin) in combination and MIC of Abietic acid (or Methicillin) alone. The FIC index values were interpreted as follows: ≤0.5, synergistic; >0.5 to ≤1.0, additive; >1.0 to ≤2.0, indifferent; and >2.0, antagonistic effects [72].

3.4.6 RNA Isolation and Real-Time PCR.

Overnight culture of MRSP was incubated with Abietic acid alone (32 μg/mL) and Methicillin alone (10 μg/mL) or in combination for 30 minutes and 1 h. Cells were harvested by centrifugation (5,000 g for 20 min at 4 °C); the supernatant was discarded and pellets were suspended into TES buffer (10 mM Tris-Cl, 1 mM EDTA, 0.5% SDS) containing 100 μg/mL of lysostaphin (Sigma-Aldrich). The control samples without compound were treated in a in a similar manner. Following incubation at 37 °C for 10 min, a Qiagen RNeasy Maxi column was used to isolate total bacterial RNA, in accordance with the manufacturer's instructions. DNA contamination was removed from the total RNA preparations using column on-RNase-Free DNase treatment (Qiagen, Germany). A_{260}/A_{280} ratio, measured by spectrophotometer (Evolution 300BB, Thermoelectro Corporation, Madison, WI, USA), was used to assess the extracted RNA purity while generalized RNA degradation was assessed by loading RNA onto an RNase-free 2% agarose gel. All amplification reactions were performed on a ABI 7500 FAST instrument (Applied Biosystems, Foster City, CA, USA) in a total volume of 20

microliter. Genes names and primer sequences used in the real-time RT-PCR analysis are listed in Table 2. The SYBR Green PCR was performed using the SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). PCR mixture included 4 µl of extracted DNA and 200 nM of primers. PCR protocol included an initial denaturation step at 95 °C for 10 min, followed by 38 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 63 °C for 45 s. The cycle threshold (CT) was determined automatically. The data collection was performed during each annealing step. Following amplification, during melting curve analysis, the temperature increases to obtain product specific melting temperature from 65 °C to 95 °C with an increment of 0.5 °C. Melt-curve analysis was also performed to assess PCR specificity, resulting in single primer-specific melting temperatures. Three samples without a DNA were routinely included as a no template control and the constitutively expressed gene for ribosomal RNA 16s (rRNA 16s) was used as an endogenous control. All analyses were performed in triplicate. In this study, relative quantification based on the expression of a target gene versus the rRNA 16s gene was utilized to determine the transcript level changes between samples. Ct values and the qPCR were normalized to the housekeeping gene for rRNA 16s using the $2^{-\Delta\Delta Ct}$ method [73].

Genes	Sequences (5 -3)
mecA	FW: TCCACCCTCAAACAGGTGAA RW: TGGAACTTGTTGAGCAGAGGT
mecI	FW: TCATCTGCAGAATGGGAAGTT RW: TTGGACTCCAGTCCTTTTGC
mecR1	FW: AGCACCGTTACTATCTGCACA RW: AGAATAAGCTTGCTCCCGTTCA
rRNA16s	FW: CGGTCCAGACTCCTACG RW: GGAGGCAGCA
MecR2	FW: TATACCCGGGAAAGTTCGTCATTGGAATCG RW: GATCGGATCCATACGCTTGTTTCGATTAGG

Table 2: *Primer sequences (5 to 3) used for PCR and real-time PCR.*

3.4.7 Effect of Abietic acid on microbial biofilm formation

The method used to assess the activity of the compound against biofilm formation is described in the section 3.2.6.
Abietic acid was added at sub-MIC concentrations ranging from 0.25 μg/mL to 2 μg/mL.

3.4.8 Effect of Abietic acid against preformed biofilm

The method used to assess the activity of the compound against mature biofilm is described in the section 3.1.7.
Abietic acid was added to the wells at the final concentration ranging from 10 μg/mL to 128 μg/mL.

3.4.9 Quantitation of metabolic activity of mature biofilm by XTT assay

See section 3.1.8.

3.4.10 Confocal laser scanning microscopy

See section 3.1.9.

3.4.11 Statistical analysis

See section 3.1.10.

4. RESULTS

4.1 VLL-28

4.1.1 Antifungal activity of VLL-28 against Candida spp.

The antifungal activity of VLL-28 against 10 clinical isolates of *Candida* spp. and *Candida albicans* ATCC10231 (used as reference strain) is reported in Table 3. The tests revealed that, among all the strains, the planktonic cells of *C. tropicalis* were the most susceptible to VLL-28, exhibiting MIC values of 12,5 μM. In particular, the MICs values for *C. albicans, C. parapsilosis* and *C. krusei* were 25 μM, whereas *C. glabrata* planktonic cells showed highest MIC of VLL-28 with values of 50 μM.
Furthermore, the activity of VLL-28 was also investigated in terms of its minimum fungicidal concentration (MFC). Compared with the corresponding MICs, the MFC values resulted to be two-fold higher for all *Candida* strains, except *C. albicans* 80 and *C. tropicalis* strains where we observed four-fold higher values of MFC compared to MICs (Table 3).

Strains	MIC90 (μM)	MFC (μM)
C. albicans ATCC10231	25	50
C. albicans 80	25	100
C. albicans 81	25	50
C. parapsilosis 3	25	50
C. parapsilosis 10	25	50
C. tropicalis 54	12.5	50
C. tropicalis 2	12.5	50
C. glabrata 28	50	100
C. glabrata 34	50	100
C. krusei 1	25	50
C. krusei 14	25	50

Table 3. Minimal concentration of VLL-28 causing 90% inhibition of cells growth (MIC) and Minimal Fungicidal Concentration (MFC) of the 11 tested Candida strains. *MIC and MBC values shown here are the highest obtained from three independent experiments*.

4.1.2 Intracellular localization of VLL-28

A fluorescein labelled derivative of VLL-28 (VLL-28*) was used to determine the intracellular localization of the peptide in *C. albicans* and *C. tropicalis* by CLSM [74]. *C. albicans* and *C. tropicalis* were chosen to investigate the intracellular localization of VLL-28 because these two strains showed the lower MICs values. Antimicrobial activity of VLL-28* was found to be similar to that of VLL-28 [77]. *C. albicans* and *C. tropicalis*, pre-incubated with MitoTracker Orange, were treated with VLL-28* at the concentration of 12,5 μM and 25 μM respectively for 15' and 2 h. Confocal images showed that, for both strains, the green signal (VLL-28*) was uniformly localized over the cellular surface of treated cells already at 15' and did not overlap with red signal of mitochondria (Figure 6), suggesting that the peptide interacts with the fungal membranes, probably binding to the negatively charged phosphate, and that there is no association with mitochondria.

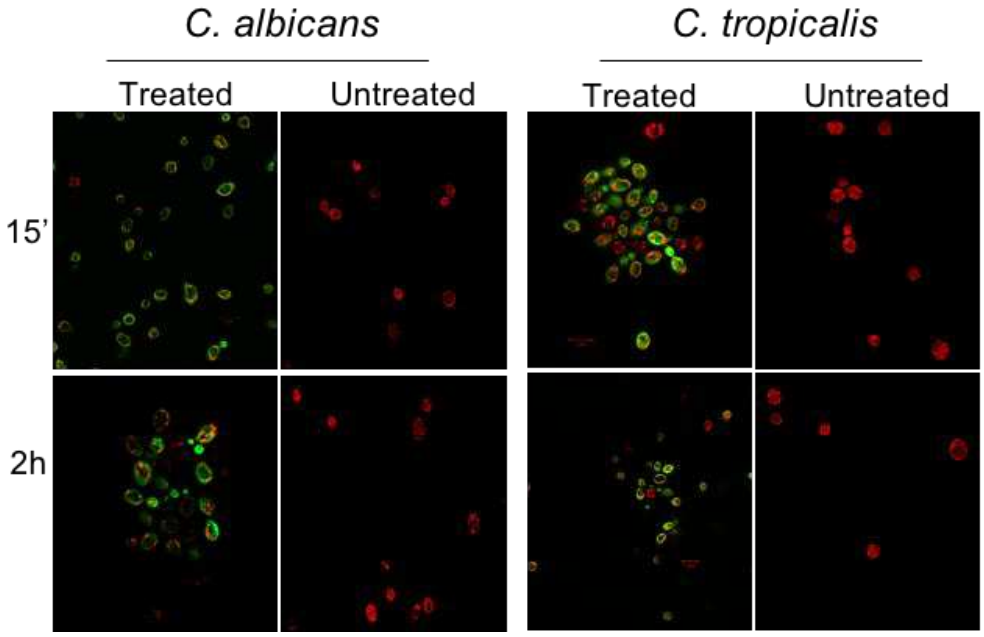

***Figure 6. VLL-28 intracellular localization by Confocal Laser Scanner Microscopy**. Candida cells were incubated in PBS with MIC dose of peptide at different timings. Treated and untreated strains are reported respectively for C. albicans and C. tropicalis. VLL-28 conjugated with FITC is coloured in green, mitotracker in red.*

4.1.3 In vitro biofilm formation assay

The biofilm producing ability was assessed by means of crystal violet assay. Results are shown in Figure 7. All strains except *C. krusei* resulted to be moderate biofilm producers at 24 h. Biofilm production increased with increasing exposure time: at 48 h, all strains were strong biofilm producers with the exception of *C. krusei* strains where only a small increase was observed.

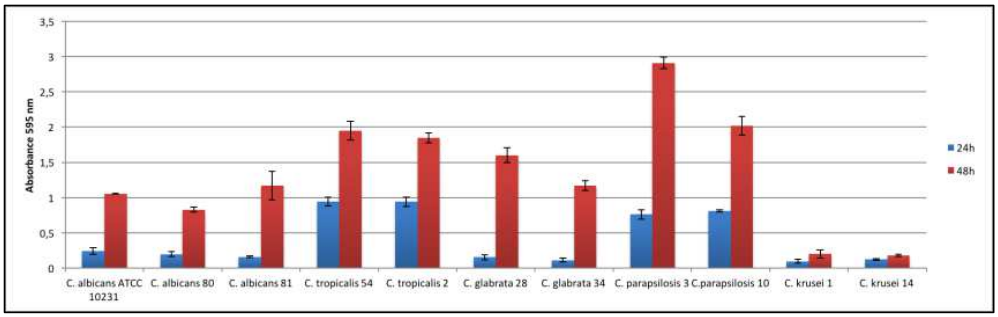

Figure 7. In vitro biofilm production levels*. Candida strains were allowed to grow in 96-well microtiter plates, and the biofilms were quantified by staining with crystal violet at 24 h (blue bars) and 48 h (red bars). Each bar represents the average, computed over three independent experiments, ± one standard deviation.*

4.1.4 Effect of VLL-28 on biofilm formation

Cell adhesion is the first step in *Candida* biofilm formation on host cell surfaces and/or abiotic substrates and plays a major role in infections. Therefore, the ability of VLL-28 to prevent biofilm production was investigated by inhibiting adhesion phase at sub-MIC levels in each strain. The minimum concentration of peptide inducing an 80% reduction of the biofilm formation (MBIC) with respect to control samples, was determined by crystal violet assay (Figure 8). The comparison of treatments with the respective controls revealed a significant strong reduction in the percentage of biofilm biomass for *C. albicans* and *C. parapsilosis* strains, with MBIC values of 12.5 µM, and for *C. glabrata* strains, with MBIC values of 25 µM. A minor reduction in the percentage of biofilm biomass was observed for *C. tropicalis*, while no inhibitory effects on biofilm formation were observed for *C. krusei* strains.

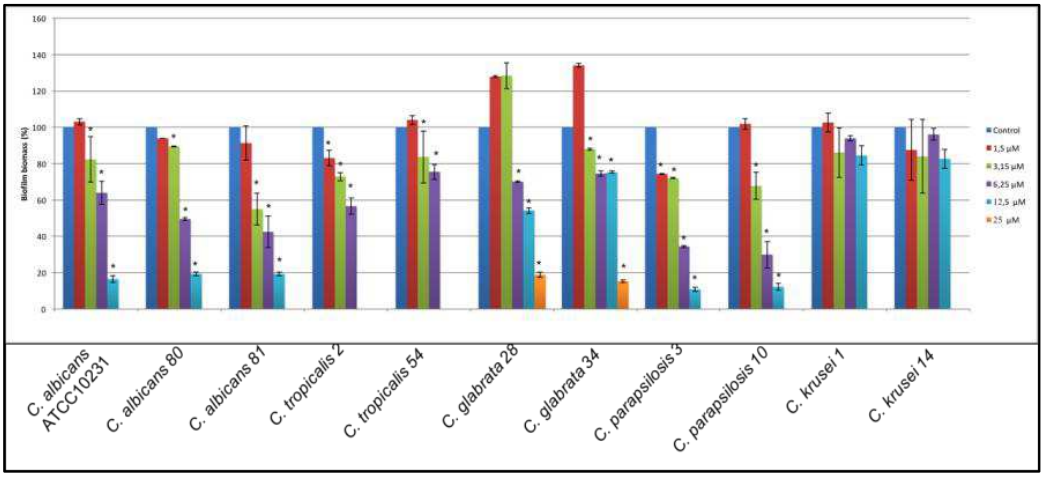

Figure 8. Effects of different concentrations of Vll-28 on forming biofilm by Candida strains. *Candida cells were incubated in RPMI 1640 in microtiter plates for 60 min with sub-MIC doses of peptide and then washed with PBS to remove non-adherent cells. The attached cells were further incubated in RPMI for 24h at 37°C. The biofilm biomass was measured by crystal violet assay. Results are reported as the percentage biofilm relative to control biofilms formed without VLL-28 treatment. For each strain, bars report average percentage (± one s.d.) of treated biofilm biomass with respect to the corresponding untreated samples. Doses are reported with different colors. Experiments were performed in triplicate in three independent experiments. For each strain, the statistical significance in average percentage of vitality between each treatment and the corresponding control was determined using T-student test (no asterisk P >0.05, *P <0.05).*

4.1.5 Activity against preformed biofilms of Candida strains

Biofilm mode of growth confers to *Candida* increased resistance to antifungal agents. For this reason, the investigation on the anti-biofilm activity of VLL-28 was also extended to preformed *Candida* biofilms. It's worth to note that, the peculiar nature of a biofilm makes it difficult to study the effect of antimicrobial agents on the bacterial community. For example, an antimicrobial agent could kill the cells inside the biofilm without affecting the extracellular matrix and hence its macroscopic structure. On the contrary, other antimicrobial agents could induce the "disaggregation" of the cells without killing them. To evaluate biomass biofilm and vitality respectively, one-day-old-biofilms were exposed at different doses of peptide that were higher than the corresponding MICs and the anti-biofilm activity was quantified using crystal violet assay and XTT reduction assay. VLL-28 was found to be able to reduce the viability of mature biofilms of almost all tested *Candida* strains, with an

$MBEC_{50}$ and $MBEC_{80}$ of 100 µM (Figure 9 – top panel). In particular, at 100 µM concentration, VLL-28 destroyed the 80% of preformed biofilm in all *C. albicans* strains, and the 50% of preformed biofilm in *C. glabrata and C. parapsilosis* strains, whereas the biofilm biomass for these two species is the same as the respective controls (Figure 9 – bottom panel). VLL-28 had a smaller effect on mature biofilm vitality of *C. tropicalis* strains, but considerably decreased its biofilm biomass. VLL-28 did not show any effect on the biofilm vitality (purple lines in Figure 9 – top panel) and biomass biofilms of *C. krusei* biofilm at all tested concentrations (last two groups in Figure 9 –bottom panel).

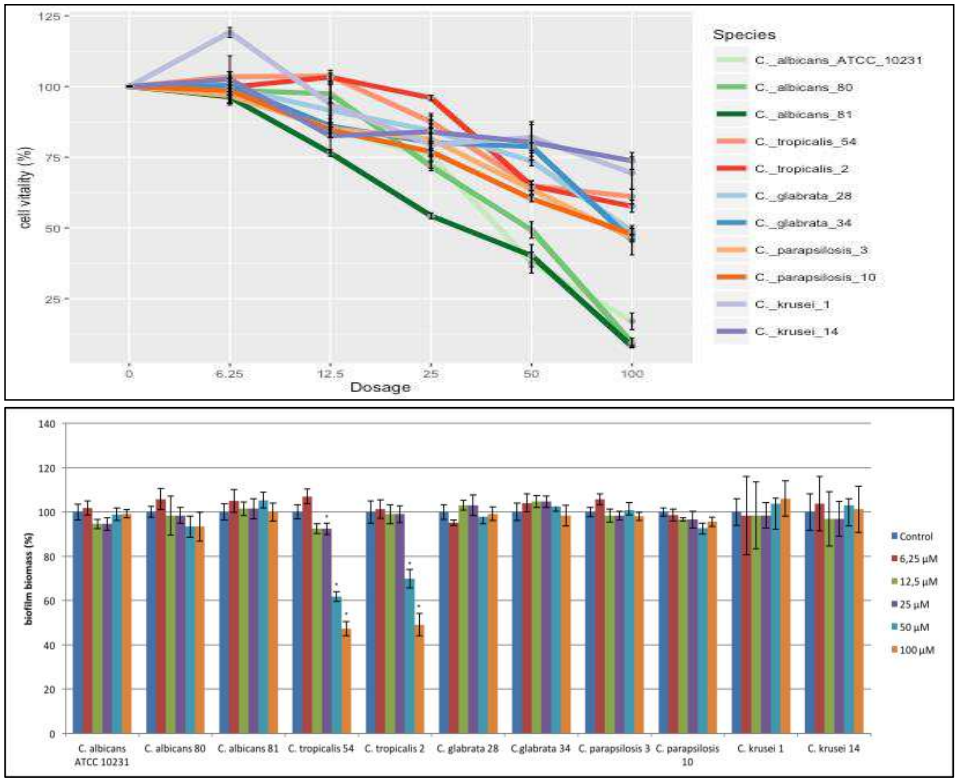

Figure 9. Effect of VLL-28 on preformed Candida biofilms. *Biofilms were grown for 24 h in 96-well microtiter plates. Mature biofilms were exposed to the peptide at concentrations ranging from 6.25 µM to 100 µM for 24 h. Experiments were performed in triplicate in three independent experiments. Top panel. Cell viability: each data point shows the average percentage of Candida treated biofilm vitality (± one s.d.) with respect to the corresponding untreated samples. Different line colours represent different strains. Bottom panel. Biofilm biomass: each bar represents the biofilm percentage variation (± one s.d.) of biomass respect to the corresponding controls (untreated). Bar colours represent different doses. * indicates significant (T-student test* $p < 0.05$*) difference compared with corresponding untreated controls.*

CLSM was used to capture the effect of VLL-28 on preformed biofilms. Biofilms were grown on Nunc® Lab-Tek® II chambered cover-glasses (Sigma) and stained with LIVE/DEAD FungaLight Yeast Viability Kit - Molecular Probes. As shown in Figure 10 by the differential staining with SYTO9 (green fluorescence, live cells) and propidium iodide (red or yellow-red fluorescence, dead cells), VLL-28 caused disruption of mature biofilms of *C. albicans, C. parapsilosis* and *C. glabrata* strains. Images show a decreased cells viability and thicknesses reduction of treated biofilms compared to controls at 48 h. Moreover, VLL-28 appears to have also a fungistatic effect on preformed biofilm of *C. tropicalis* strains; indeed, the thickness of treated biofilm was the same as the respective control at 24 h but there was no dead cell. Results seem to suggest that even if the peptide isn't able to kill *C. tropicalis* cells embedded in biofilm, it is capable to arrest its biofilm growth. Finally, exposure to VLL-28 did not induce any visible change in thicknesses and vitality of the *C. krusei* biofilm. CLSM images confirmed results obtained by XTT reduction assay.

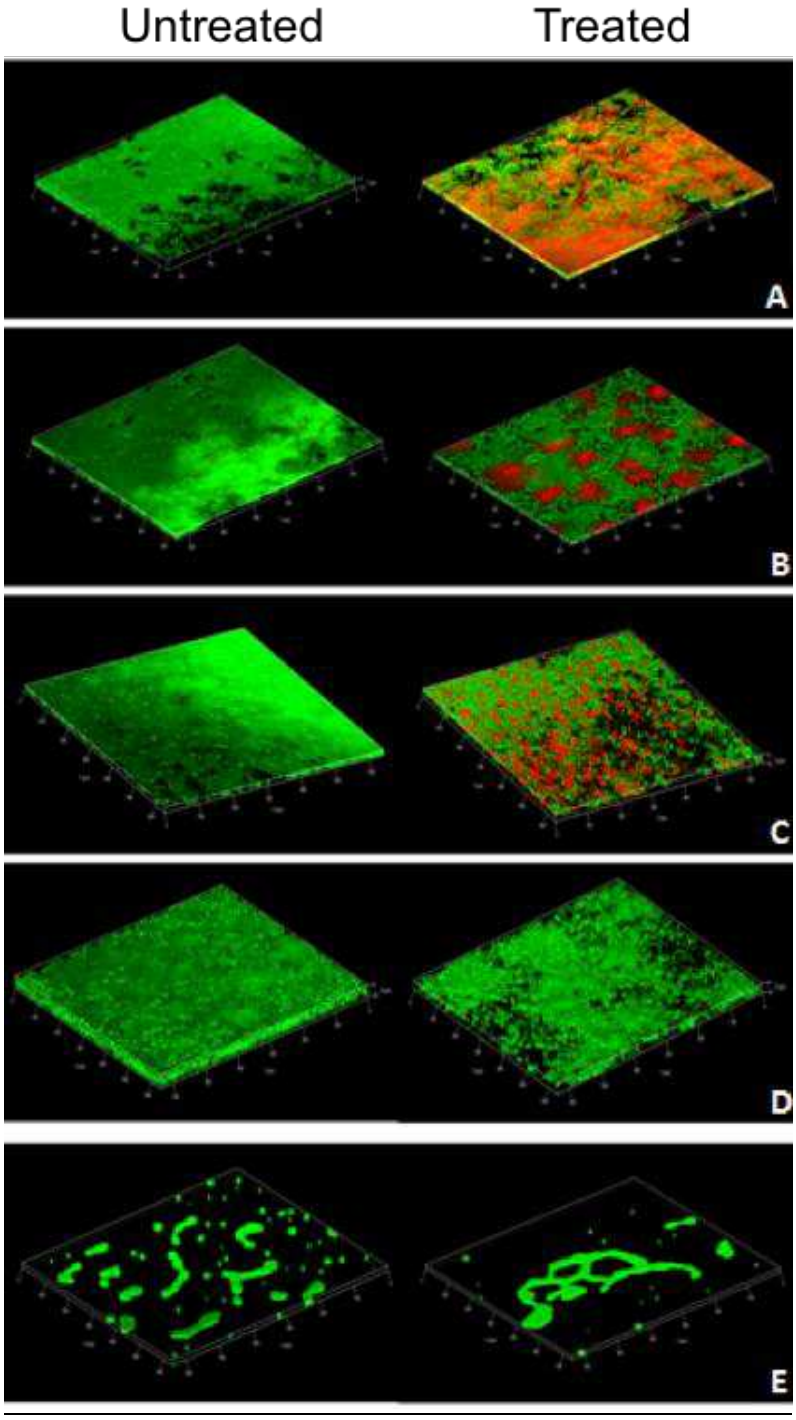

Figure 10. Three-dimensional analyses of effect of VLL-28 on preformed biofilm using confocal microscopy. *Biofilms were grown in static condition. VLL-28 at 2xMIC concentration, was added on a 1-day-old biofilm for 24 h. Cells were stained in green with Syto-9 and red with the "dead" stain propidium iodide.* ***A)*** *C. albicans* ***B)*** *C. parapsilosis;* ***C)*** *C. glabrata;* ***D)*** *C. tropicalis****; E)*** *C. krusei.*

4.2 Human pepsinogen derived peptides

4.2.1 In silico identification of human pepsinogen derived peptides

The computational tool, described in [68], for the identification of cryptic AMPs is based on a score, called the "absolute score" (AS). This value, derived from the amino acidic composition of an AMP and a strain-dependent variable, is linearly correlated with the antimicrobial potential of the given AMP. This tool was used to analyze mammalian zymogens, in particular human aspartic protease, and found three potential AMPs located in the N-terminal region of human pepsinogen isoform A3, corresponding to the pepsinogen activation peptide. According to the parameter set used, AS values between 6.0 and 7.6 correspond to MIC values in the range of 160-10 μM, whereas AS values higher than 7.6 correspond to MIC values lower than 10 μM. For AS values close to or higher than 9, the linear correlation is no longer valid, so that further increase of the AS values is unlikely to correspond to any decrease of MIC values. The fragment at the N-terminus of human pepsinogen A3, corresponding to pepsinogen A activation, contains two high-scoring fragments with AS values higher than 9 (Figure 11). The full-length PAP-A3 activation peptide (here named PAP-A3) showed an AS values of 12.7. The isometric plots also show that the fragment 1-24 corresponding to the N-terminus region of PAP-A3 (here named IMY25) has a local maximum with AS = 8.9 suggesting significant antimicrobial activity, instead the fragment 26-47 corresponding to C-terminus region of PAP-A3 (named FLK22) has an AS value slightly higher than the lowest threshold (AS = 6.1), suggesting a possible, even if low, antimicrobial potential. It is worth noticing that these two small peptides (IMY25 and FLK22) may be released *in vivo* as a consequence of PAP-A3 hydrolysis at Asp25-Phe26 in the acid stomach lumen [75].

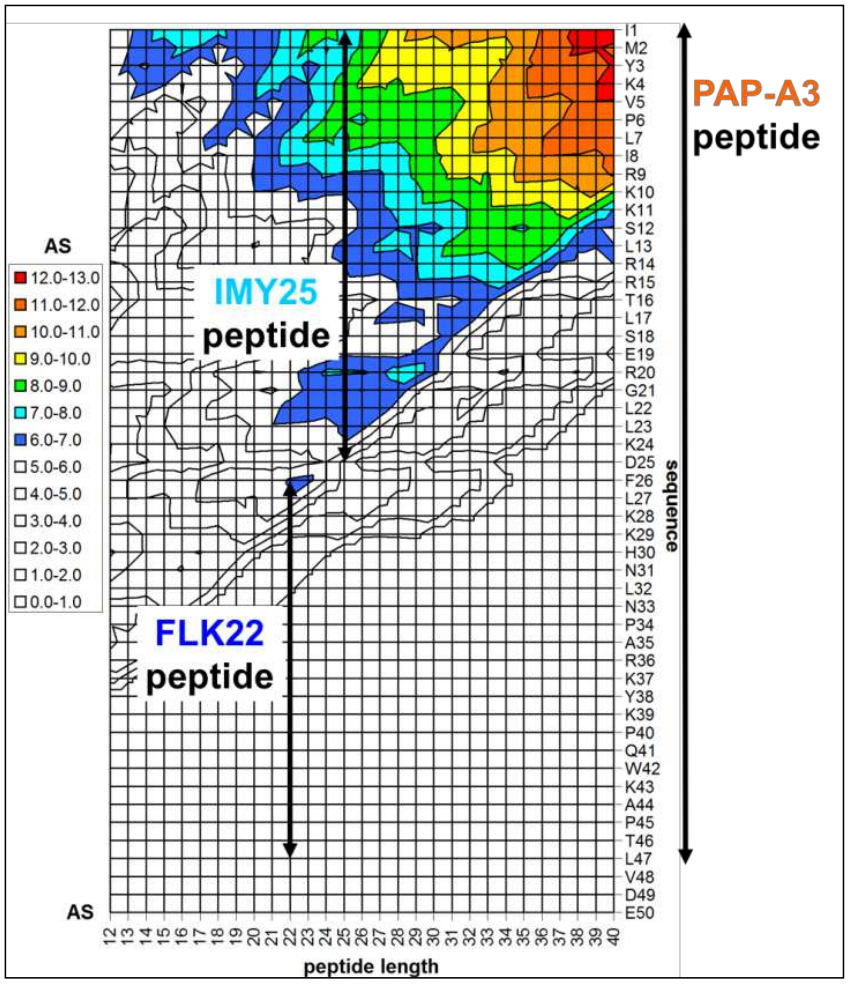

Figure 11. Computational tool for detection of cryptic AMPs in human pepsinogen isoform A3. *Analysis of the absolute scores (AS) of the peptides in the N-terminus of pepsinogen A3. AS values were calculated using the parameters determined for strain S. aureus C623 and the hydrophobicity scale Parker-Gly0. Peptide length is shown on the x axis. Protein sequence (residues 1-50) is shown on the y axis. Colours highlight scores corresponding to hypothetical MIC values. In the range 6 < AS < 10, AS values are linearly correlated to MIC values. AS > 6 corresponds to a hypothetical MIC lower than 160 µM. AS > 7.6 corresponds to a hypothetical MIC lower than 10 µM. AS > 9 are unlikely to correspond to a decrease of MIC values.*

Pepsin A is secreted by chief cells as an inactive zymogen, pepsinogen A, containing at the N-terminus a prosegment also known as activation peptide (47 residues long in the case of the human protein) [75]. The activation peptide, spanning the active site of pepsin A, has two important functions: it stabilizes the folding of pepsin A at neutral pH (pepsin A undergoes denaturation at pH close to 7 or higher), and it inhibits potentially dangerous proteolytic activity. After secretion, the acidic pH of gastric juice promotes

zymogen activation through cleavage of activation peptide from pepsinogen A. This process can be autocatalytic (i.e., intramolecular) or mediated by an already functional pepsin molecule (intermolecular). Moreover, activation can occur in a single step, releasing the 47-residue peptide, or in two steps, with an intermediate cleavage in the middle of the activation peptide (e.g., between either Leu23 and Lys24 or Asp25 and Phe26 in the case of human pepsinogen A). Low pH values (below 2) favor the intramolecular process, whereas high concentration of pepsin A and high pH values favor the intermolecular process and single-step activation [75].

4.2.2 Recombinant production of pepsinogen A3 derived peptides

Following results of the previously reported analysis, a new carrier, derived from a modified ribonuclease, was used to produce a recombinant activation peptide in *E. coli* as a fusion protein. The engineered carrier enables the production of AMPs in *E. coli* as insoluble fusion proteins (inclusion bodies) in which peptides are located at the C-terminal side. Carrier (N-terminal region) and peptides (C-terminal region) are joined by a flexible linker region in which an Asp-Pro acid labile site was located to allow chemical release of recombinant peptide from the carrier. This well described cleavage method [76] leads to the production of peptides with an additional proline residue at the N-terminal end of the recombinant peptide.
ONC-DCless-H6-(P)PAP-A3 fusion protein was used to produce the recombinant full length PAP-A3 activation peptide, named (P)PAP - A3 due to the additional N-terminal proline residue.
Two shorter recombinant peptides, named IMY25 and FLK22, were produced from ONC-DCless-H6-(P)PAP-A3(Pro26) fusion protein.
The recombinant protein was very similar to ONC-DCless-H6-(P)PAP-A3 protein, except for the insertion of a proline residue between Asp25-Phe26 residues. In this way, a second acid labile site was introduced inside the full-length activation peptide. Chemical cleavage of the fusion protein led to simultaneous production of both shorter recombinant peptides, named (P)IMY25 and (P)FLK22 (Figure 12).

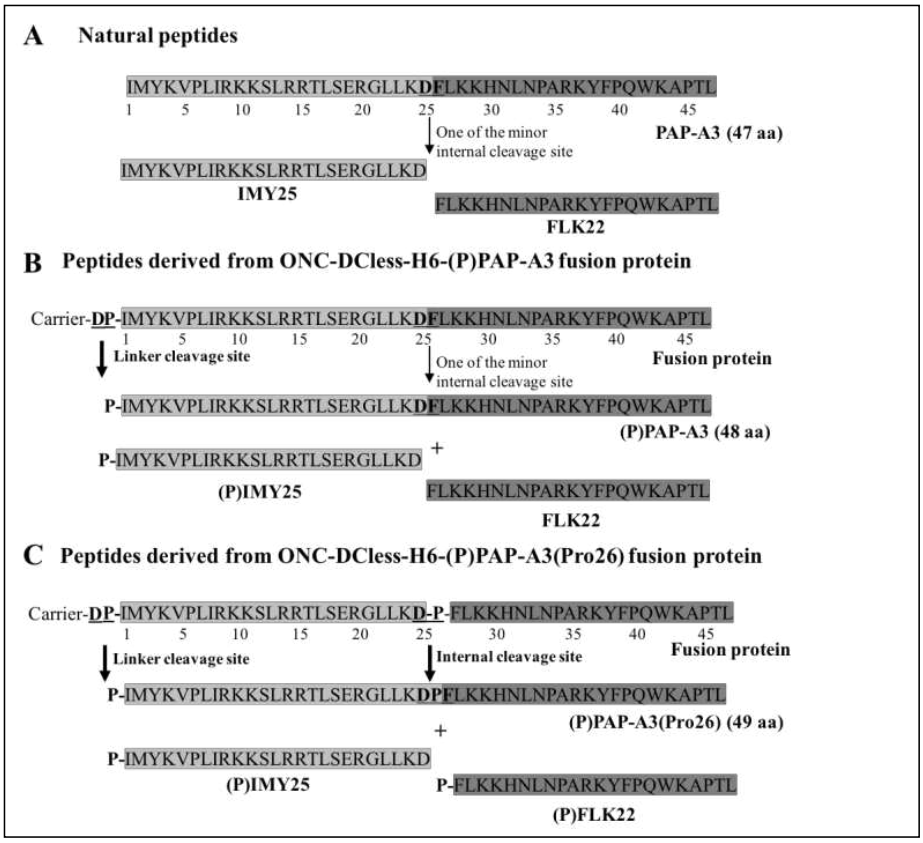

Figure 12. Natural and recombinant peptides derived from human pepsinogen A3 activation peptide. ***A)*** *Full length pepsinogen activation peptide and its two shorter peptides derived from cleavage of DF internal acid labile site (bold and underlined characters) into the acid stomach environment.* ***B)*** *Recombinant peptides derived from ONC-DCless-H6-(P)PAP-A3 fusion protein by cleavage of DP site into the protein linker region [(P)PAP-A3 peptide] and DF internal cleavage site [(P)IMY25 and FLK22 peptides].* ***C)*** *Recombinant peptides derived from ONC-DCless-H6-(P)PAP-A3(Pro26) fusion protein by cleavage of DP site into the protein linker region [(P)PAP-A3(Pro26) peptide] and DPF internal cleavage site [(P)IMY25 and (P)FLK22 peptides]. Internal cleavage sites were highlighted as bold and underlined characters.*

Fusion proteins were expressed at high levels in the inclusion bodies in *E. coli* BL21(DE3) strain and purified by IMAC as described in [77]. Release of peptides from recombinant proteins was obtained by chemical cleavage of Asp-Pro sequences in acidic environment (pH 2). The SDS-PAGE analysis (Figure 13) shows that, after 24 h of incubation at 60°C, the Asp-Pro sequences situated in the linker region of both fusion proteins, was efficiently cleaved (about 95% efficiency). Peptides were purified from the carrier by selective precipitation of the carrier at pH 7.2, followed by repeated cycles of centrifugation. As shown by SDS-PAGE analysis in Figure 13,

peptides were recovered in the soluble fraction at neutral pH, whereas carriers were found to be in the insoluble fractions.

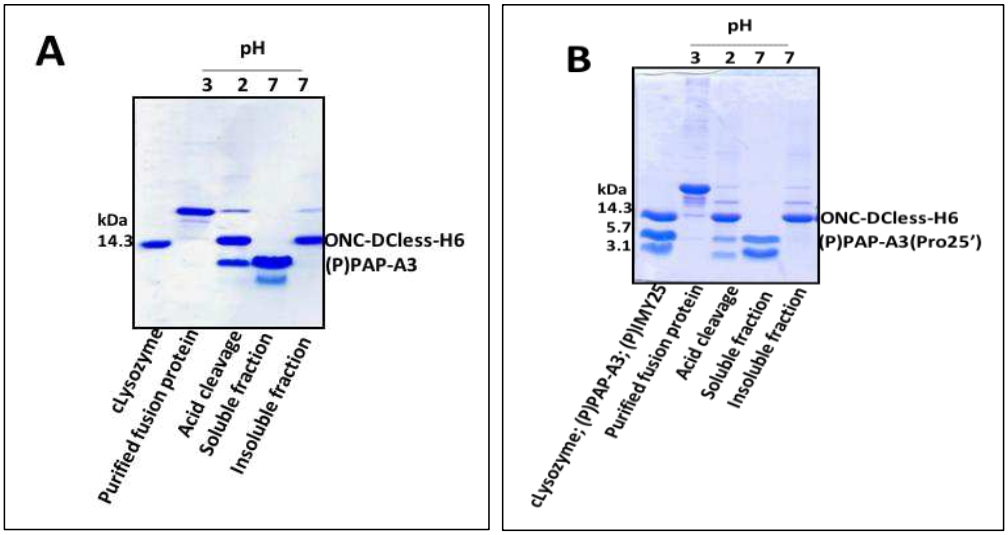

Figure 13. Products deriving from the acid catalyzed hydrolysis of ONC-DCless-H6-(P)PAP-A3 and ONC-DCless-H6-(P)PAP-A3(Pro25') fusion proteins*. SDS-PAGE analysis of the hydrolysis products of ONC-DCless-H6-(P)PAP-A3 (Panel **A)** and ONC-DCless-H6-(P)PAP-A3(Pro25') (Panel **B)**, respectively before and after the selective precipitation of the ONC carrier at pH 7. In both panels, lane 1, markers; lane 2, fusion proteins before the acidic hydrolysis; lane 3, fusion proteins after the acidic hydrolysis; lanes 4 and 5, soluble and insoluble fraction, respectively, obtained after the selective precipitation at pH 7.*

RP-HPLC analyses of the peptide mixtures derived from acid cleavage (Figure 14), highlighted three main peaks in each mixture. Mass spectrometry analyses revealed that the 5% of the ON-DCless-H6-(P)PAP-A3 fusion protein was cleaved in the site inside the full length pepsinogen releasing (P)IMY25 and FLK22, while the 80% of the ON-DCless-H6-(P)PAP-A3(Pro26) fusion protein was cleaved to produce the two short peptides.

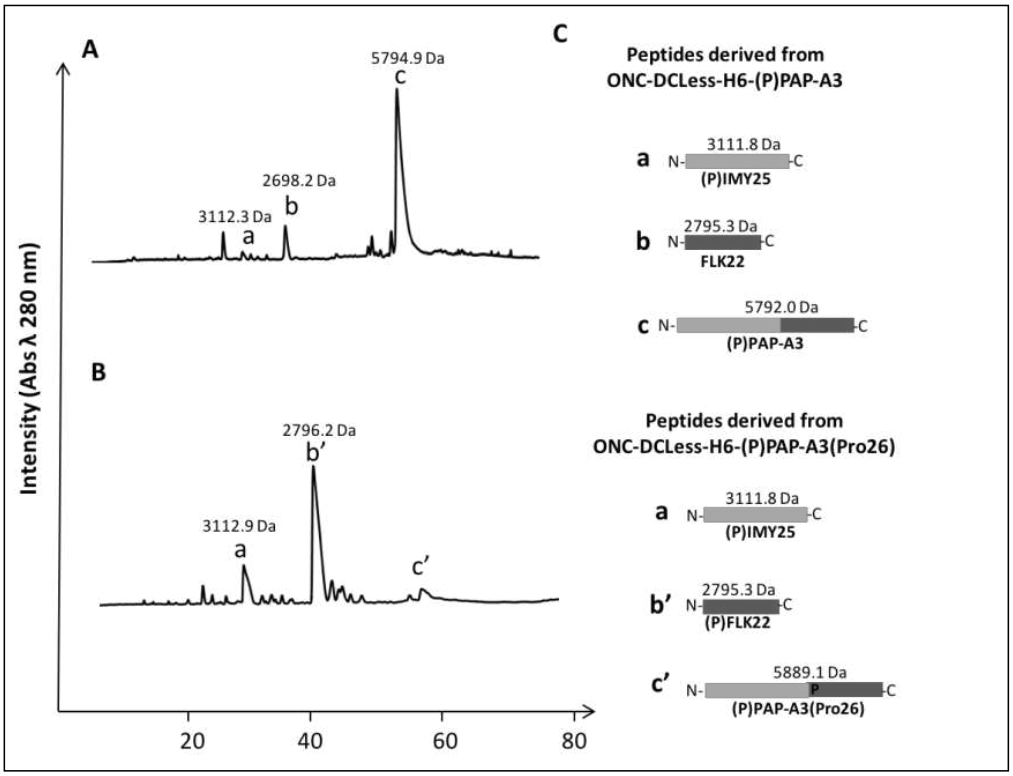

Figure 14. ***RP-HPLC analyses of peptide mixtures.*** *Samples were analysed after selective carrier precipitation at neutral pH on Jupiter 5u C18 300A column.* ***A)*** *Peptides derived from cleavage of ONC-DCLess-H6-(P)PAP-A3 fusion protein; peak c, (P)PAP-A3 full length peptide; peaks a and b, the two shorter peptides [(P)IMY25 and FLK22, respectively] derived from cleavage of DF acid labile internal site.* ***B****) Peptides derived from cleavage of ONC-DCLess-H6-(P)PAP-A3(Pro26) fusion protein; peak c', (P)PAP-A3(Pro26) full length peptide; peaks a and b', the two shorter peptides [(P)IMY25 and (P)FLK22, respectively] derived from cleavage of DPF acid labile internal site. Molecular weight of peptides determined by Mass spectrometry were reported on the chromatograms.* ***C)*** *Representation of all recombinant peptides and their theoretical molecular weights. (at chromatograms of recombinant peptides following acid cleavage and carrier selective precipitation at pH 7.2 derived from ONCDCLessH6-(P)PAP -A3 (A) and ONCDCLessH6-(P)PAP-P26* (B). Peptides expected from each fusion protein and their theoretical molecular weight obtained by Ptotparam tool (C). Peptides indicated as b and b' differs only for an additional proline at the N-terminus. Peptides indicated as c and c' differs only for an additional proline inserted downstream Asp25.)*

(P)PAP-A3 full length peptide was produced with a final yield of about 18-20 mg/L, whereas (P)IMY25 and (P)FLK22 shorter peptides gave 7-8 mg/L of culture final yield.

4.2.3 Antibacterial activity

Pepsinogens are pro-enzymes released by chief cells that are present within the stomach wall, and are activated into aspartic protease pepsins after being mixed with the hydrochloric acid of the gastric juice. Thus, after the production of (P)PAP-A3, (P)IMY25 (corresponding to the recombinant N-terminus region of PAP-A3) and (P)FLK22 (corresponding to the recombinant C-terminus region of PAP-A3), their potential antimicrobial activity was evaluated against a wide panel of Gram-negative and Gram-positive bacteria including gut bacteria, clinical multidrug-resistant strains and biofilm forming strains.
As shown in Table 4, with the exception of *Pseudomonas aeruginosa* and *Listeria monocytogenes* isolates, (P)PAP-A3 exhibited broad-spectrum antimicrobial activity against all tested bacterial strains (MIC value $\leq$ 12.5 μM). In addition, recombinant PAP-A3 showed higher antimicrobial potency than the individual shorter peptides. On the other hand, (P)IMY25 peptide inhibited the growth of most bacteria, but its MIC values were higher than the *full-length* molecule. Finally, (P)FLK22 exhibited weaker antimicrobial activity and was active only on few strains.
In vivo, IMY25 and FLK22 are released in stoichiometric amounts from pepsinogen A as a consequence of the internal hydrolysis occurring in the middle of the prosegment. For this reason, the antibacterial properties of an equimolar mixture of (P)IMY25 and (P)FLK22 were also investigated. Intriguingly, results indicate that for all tested strains, the mixture did not possess the same antibacterial effect exerted by the *full-length* peptide, but almost preserves the entire activity of the (P)IMY25 peptide alone. These results indicate that it's likely that even if the precursor would lose its integrity, the antibacterial activity would be, at least in part, retained.

Strains	(P)PAP-3A	(P)IMY-25	(P)FLK-22	Mix
E. coli ATCCATCC25922	6.25	25	>50	25
E. faecalis ATCC29212	12.5	50	>50	50
S. enteriditis 706RIVM	6.25	25	>50	50
L. monocitogenes	>50	>50	>50	>50
S. typhimurium ATCC14028	6.25	25	>50	12.5
P. aeruginosa RP73	6.25	6.25	12.5	12.5
P. aeruginosa AA2	>50	>50	>50	>50
P. aeruginosa KK27	>50	>50	>50	>50
P. aeruginosa PAO1	>50	>50	>50	>50
S. aureus MRSA	6.25	25	>50	25
S. aureus ATCC6538P	1.56	1.56	3.12	25
A. baumannii ATCC17878	1.56	nd	nd	nd
B. spizizenii ATCC6633	12.5	nd	nd	nd
K. pneumoniae ATCC700603	6.25	nd	nd	nd
P. aeruginosa ATCC27853	12.5	nd	nd	nd

Table 4. Antibacterial activity of the recombinant pepsinogen A3 derived peptides. *Assays were carried out by broth dilution method in Nutrient Broth 0.5 X. Reported MIC values (μM) are the highest obtained in each one of three independent experiments*.

4.2.4 Time killing assay

The MIC assay reported in the previous section does not discriminate between bactericidal and bacteriostatic activity of peptides. Moreover, it cannot be performed at low pH values, which inhibit bacterial growth. To address these points, a plate viable count assay was performed on *Salmonella enterica* serovar *enteriditis*, *Salmonella enterica* serovar *typhimurium* and *Escherichia coli*, three common intestinal bacteria. Strains were diluted in nutrient broth at pH 7. Part of the cultures was acidified to pH 3.5 by HCl addition to mimic the stomach environment, (P)PAP-A3 was added at a physiological concentration (10 μM). Moreover, to mimic the transfer from stomach to duodenum, samples were tested at pH 7 as well. After different time treatments, samples were plated to count the number of living cells. As shown in Figure 15, within 45 minutes at pH 7, (P)PAP-A3 significantly reduced the number of viable cells of all strains. While *E. coli* did not survive itself at pH 3.5, (P)PAP-A3 was able to kill *Salmonella* species, that are more resistant to acidic pH levels. Indeed, *S. typhimurium* survival percentage was reduced by ~40% within 15' upon treatment at pH 3.5 (Figure 15 – panel B).

From these analyses, it can be concluded that (P)PAP-A3 possesses bactericidal activity at both neutral and acidic pH values.

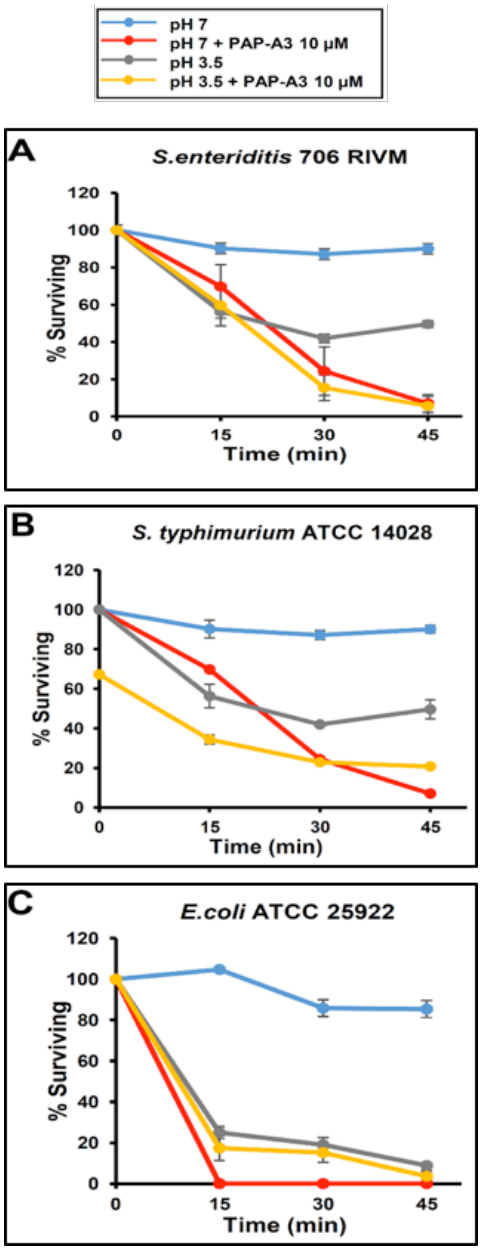

Figure 15. Time-kill assay of recombinant pepsinogen activation peptide PAP-A3. *Stationary phase bacteria were diluted in Nutrient Broth 0.5 X. Aliquots were taken at specified time points, serially diluted into Nutrient Broth 0.5 X with 5 mM sodium hydrogen carbonate in order to neutralize the pH. CFU were determined after overnight growth on plates. Different line colours represent different growth conditions. Experiments were performed twice independently. Error bars denote standard deviation from the mean.*

4.2.5 Determination of the minimum inhibitory concentration and minimal bactericidal concentration like-biofilm-condition

The efficient formation of a biofilm for all tested species required the addition of glucose to the medium. The antibacterial activity test was therefore repeated in the biofilm conditions in order to check if the addition of glucose (0,5%) to the medium could have altered the growth inhibition activity of the tested peptides. The peptides inhibited the growth of *S. aureus* ATCC6538P at the same MIC values of experiments performed without glucose, and had no effect on *P. aeruginosa* PAO1 growth, revealing that the addiction of glucose does not alter peptides action. The bactericidal and bacteriostatic effects of the three peptides was also investigated. PAP-A3, (P)IMY25 and (P)FLK22 had a bacteriostatic effect on *S. aureus* growth at the MIC values. At 10 μM (P)PAP3A showed a bactericidal effect, while (P)IMY25 and (P)FLK22 showed a bacteriostatic effect at higher concentrations than MIC values.

4.2.6 Effect on biofilm formation

To further evaluate the role in host defence of PAP-A3 and its fragments, their efficacy against the formation of bacterial biofilms was tested. Biofilm formations of *P. aeruginosa* PAO1 and *S. aureus* ATCC 6538P were found to be resistant to inhibition by all the PAP3A derived peptides. In fact, no significant alterations in the percentage of biofilm biomasses were observed after treatment at different doses (Figure 16 – panel A). The effects of the tested peptides on biofilms formation were also assessed by means of confocal laser scanner microscopy following staining of 24 h old biofilm with Syto9 and propidium iodide (Figure 16 – panel B). Acquired images of treated biofilms did not show any significantly change in the structure or number of cells respect the relative untreated biofilms. These results confirm the previous data.

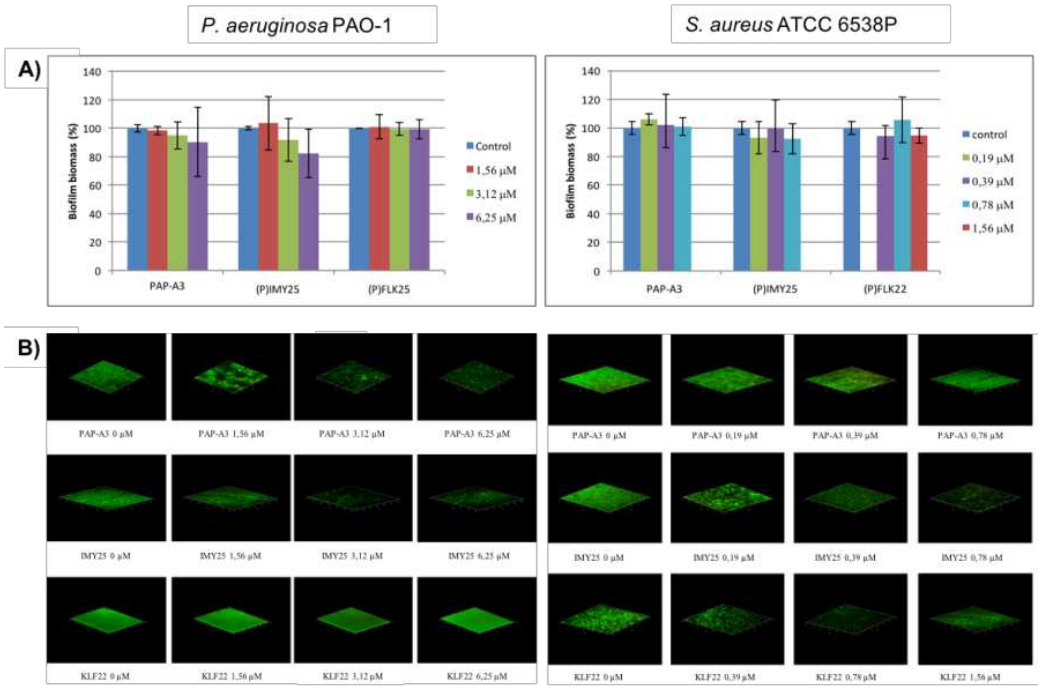

Figure 16. Activity of (P)IMY (1-47), (P)IMY (1-25) and (P)FLK (26-47) on biofilm formation. ***A)*** *Biofilm biomass percentage measured by crystal violet. Bars represent the percentage (± one s.d.) of biomass formation with respect to the corresponding controls (untreated). Bar colours represent different doses. Each experiment is representative of three independent tests each one performed in triplicate.* ***B)*** *Three-dimensional images acquired by CLSM. Up, middle and bottom panel show untreated and treated biofilm and biofilm with (P)PAP-A3, (P)IMY25 and (P)FLK22 at different concentrations.*

4.2.7 Pepsinogen A3 peptides activity against preformed biofilm

In order to assess the anti-infective potential of the three pepsinogen-derived peptides, their efficacy against mature bacterial biofilms was tested. To determine the anti-biofilm properties of PAP-A3 derived peptides the total biofilm biomass and cell viability were measured in multiwell plates by crystal violet staining and the XTT assay, respectively. On the other hand, CLSM was used to see the effect of the peptides on the structure and viability of biofilms obtained in two different experimental setups: a static biofilm growth chamber, and a flow cell-based system. The main distinction between these two systems is the use of continuous (and constant) flow in the flow cell apparatus, which enables passive motion of biomass and prevents excessive binding of compounds to abiotic surface and/or extracellular biofilm matrix components [78].

First, the ability of peptides (P)PAP-A3, (P)IMY25 and (P)FLK22 to eradicate preformed biofilms of Gram-positive *S. aureus* ATCC6538P strains was assessed by means of a crystal violet assay and standard XTT cell viability assay.
Crystal violet data and CLSM micrographs (Figure 17 – top panel) revealed that the peptides did not reduce biofilm biomass with any of the tested concentrations. On the other hand, (P)IMY25 reduced cell viability of pre-established biofilms after 24 h of treatment in a concentration-dependent mode (6.25 μM, 12.5 and μM, 25 μM) (Figure 17 – bottom panel). The highest level of (P)IMY25 induced cell death and were observed at a concentration of 16X MIC (25 μM). In addition, *S. aureus* ATCC6538P resulted unreactive in the XTT cell viability assay upon treatment with (P)IMY25. This may plausibly correlate to the extreme variation of metabolic activity exhibited by *S. aureus* isolates [79]. Surprisingly, (P)FLK22 and (P)PAP-A3, whose MIC value against planktonic bacteria was identical to that of (P)IMY25, did not kill biofilm cells even at the all tested concentration.

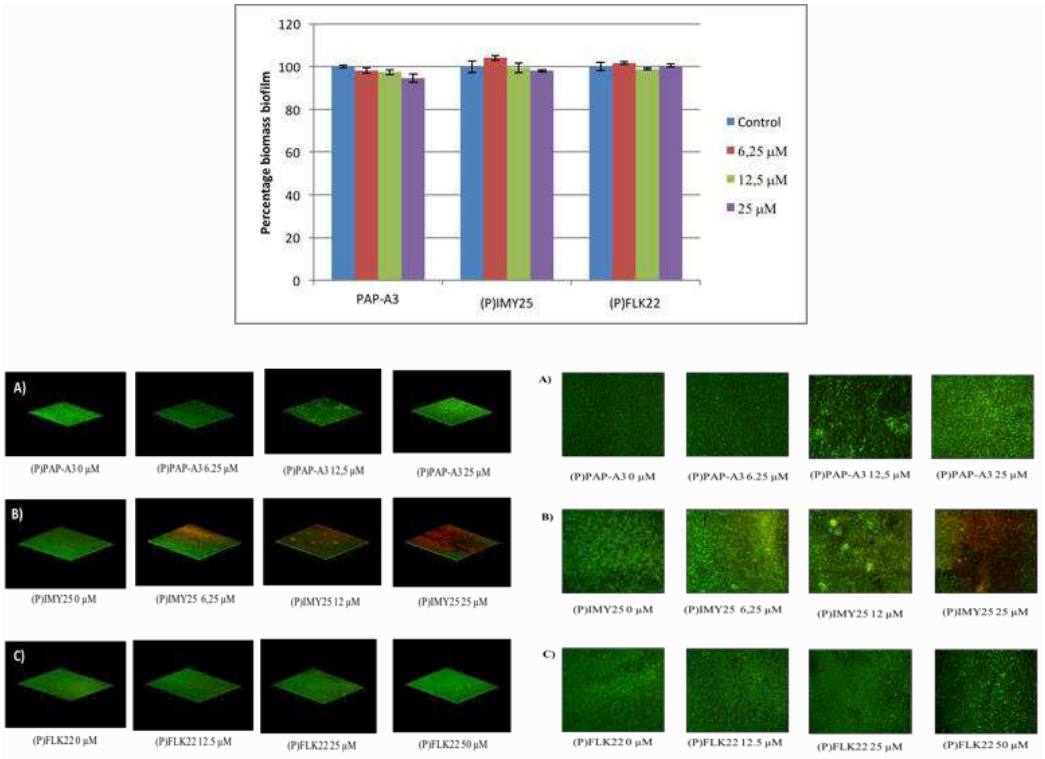

Figure 17. Effects of different concentrations of PAP-A3, (P)IMY25 and (P)FLK22 on S. aureus ATCC6538P mature biofilm. *Top panel: Bars represent the average ± one s.d. of biofilms biomass quantified through crystal violet assay. Each experiment is representative of three independent tests each one performed in triplicate. Bottom panels: CLSM images showed the anti-biofilm activity of (P)PAP-A3* ***A)****, (P)IMY25* ***B)*** *and (P)FLK22* ***C)*** *peptides at the concentration ranging from 6.25 µM to 50 µM against S. aureus ATCC6538P one-day-old biofilms. 3D images (left panels), ortho images (rights panels).*

Similar experiments were carried out using *P. aeruginosa* PAO1. At the lowest tested concentration of 6,25 µM, peptide (P)IMY25 significantly reduced *P. aeruginosa* PAO1 biofilm biomass (Figure 18 – top panels) and the viability of biofilm embedded bacteria (Figure 18 – bottom panels). Interestingly, both of the previously described effects resulted to be markedly reduced when doubling the peptide concentration, and were no longer observed with the highest tested concentration of 25 µM. In contrast, the full-length peptide (P)PAP-A3 and its derivative (P)FLK22 did not show any anti-biofilm activity at 6.25, 12 and 25 µM.
CLSM studies confirmed that, out of the three AMPs tested, (P)IMY25 exhibited the most effective biofilm disruption activity against preformed *P. aeruginosa* biofilms. (Figure 18 – bottom panel).

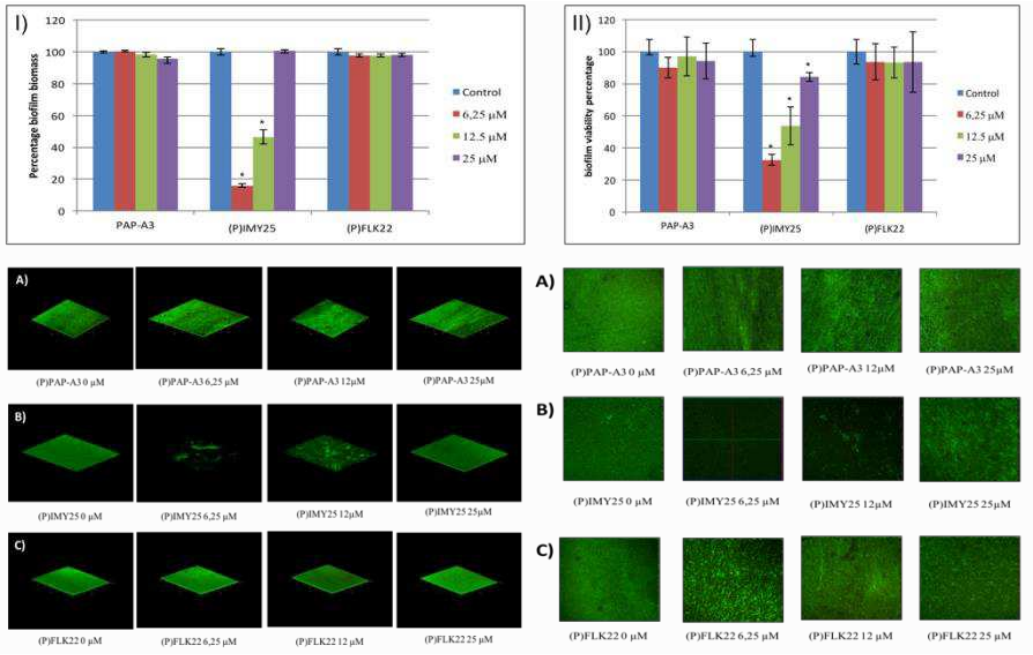

Figure 18. Effect of PAP-A3 peptides against P. aeruginosa PAO-1 preformed biofilm. *Top panels: quantification of biofilms mass performed by CV **I**) and biofilm viability by XTT reduction assay **II**). Data in percentage are means ± standard deviations. Each experiment is representative of three independent tests. Differences between each treatment and the corresponding untreated control were tested by using T-student test and a p value of < 0.05 was considered statistically significant. (no asterisk P >0.05, *P <0.05). Bottom panels: CLSM 3D micrographs and ortho images reporting the effect of peptides (P)PAP-A3 **A**), (P)IMY25 **B**) and (P)FLK22 **C**) on P. aeruginosa PAO1 one-day-old biofilms treated with different peptide concentrations ranging from 6.25 µM to 25 µM.*

These results revealed that, under static conditions, (P)PAP-A3 and (P)FLK22 did not exhibit any biofilm activity. In contrast, (P)IMY25 treatment caused a strong biofilm detachment and/or disaggregation of preformed biofilm of *P. aeruginosa* PAO1 already at very low concentrations (6.25 µM), and a notable reduction in living cells on preformed biofilm of *S. aureus* ATCC6538P. It should be remembered that *Pseudomonas aeruginosa* (a Gram negative) and *Staphylococcus aureus* (a Gram positive) are very different strains and the chemical nature of their extracellular matrix, as well as the ability to respond to signal molecules, could be very different. Therefore, the interaction of the peptide with the *P. aeruginosa* biofilm cannot be explained by the simple hypothesis that the matrix acts as a filter.

In order to obtain more details on the activity of (P)PAP-A3 and its shorter peptides against *P. aeruginosa* PAO1 biofilms, a second experimental setup based on a flow cell system coupled with confocal

microscopy was used for the visualization of biofilms. Compared to the one previously described, this system allows the bacterial biofilm to grow under continuous flow of medium, in the absence (untreated control) or presence of peptide [45]. In this condition, biomass passive transport, mediated by the constant flow rate, prevents excessive binding of compounds to abiotic surfaces or to extracellular matrix components [78]. Interestingly, results obtained using this setup, under flow chamber, show an opposite trend compared to those obtained under static conditions (Figure 19).

Here, (P)PAP-A3 exhibited the strongest anti-biofilm activity against 48 h old *P. aeruginosa* PAO1 biofilms (Figure 19-B) when compared to corresponding untreated control (figure 19-A), moreover, in this case no red staining was observed, indicating that the peptide has a detachment/disaggregation effect. (P)FLK22 (Figure 19-C), did not have a clear effect on preformed biofilms. Interestingly, under flow conditions, (P)IMY25 did not have any clear effect on biofilm mass or on the total number of cells, but the number of red-stained nonviable cells was significantly higher than in the control group or in samples treated with the other two peptides.

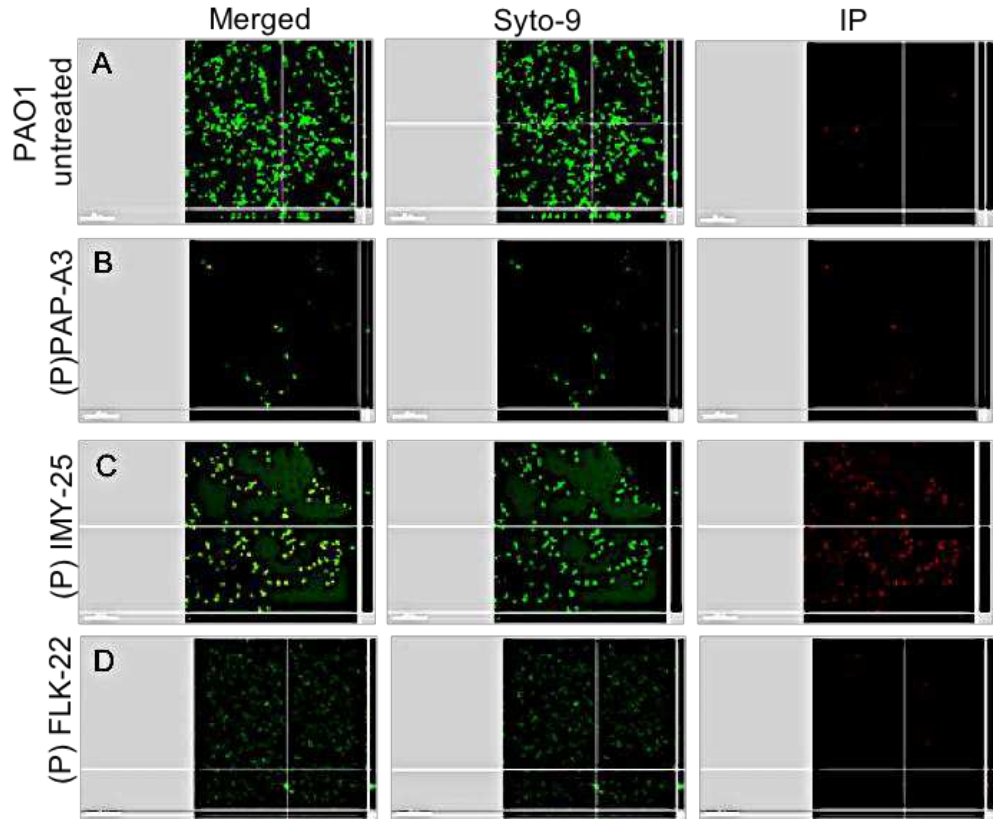

Figure 19. Effect. Effect of PAP-A3-derived peptides on P. aeruginosa PAO1 preformed biofilms grown under flow conditions. *P. aeruginosa PAO1 biofilms were cultivated in minimal medium for 48 h at 37°C in flow chambers* ***A)*** *before the addition of peptides (P)PAP-A3, (P)IMY25 or (P)FLK22* ***B)***, ***C)***, ***D)*** *respectively were added into the flow-through medium at concentration of 3 μM for each peptide. Images correspond (from left to right) to PAO1 biofilm stained with SYTO-9 (live cells), PAO1 biofilm stained with propidium iodide and merged image. Each panel shows reconstructions from the top in the large panel and sides in the right and bottom panels (xy, yz and xz dimensions). Scale bars in the bottom-left corner of each image correspond to 20μm in all the cases.*

4.2.8 Interaction with Alginate

The apparent conflict emerging from the above reported data can be explained by the fact that, in biofilms, bacteria are encapsulated in the extracellular matrix that is composed by negatively charged polysaccharides. This allows us to hypothesize that, in the absence of flow, (P)PAP-A3 may be binding to one or more components of the biofilm matrix that in turn limits its anti-biofilm activity. One of the most important component of the *P. aeruginosa* biofilm matrix is alginate [80], an acidic linear polysaccharide composed by β-D-mannuronate and α-L-guluronate residues in different proportions

and arranged either in homo-polymeric blocks or mixed blocks [81]. Several cationic AMPs are able to bind to alginate, often changing conformation upon binding. Deber and co-workers demonstrated, that alginate can induce α-helical conformation in AMPs – mimicking the effect of membrane binding– and even promote their self-association [82-83], protecting in this way matrix-embedded cells.
In collaboration with Dr. Notomista's group of the Department of Biology University of Naples Federico II, the binding ability of pepsinogen A-derived to alginate was investigated by the CD spectra of fixed concentrations of the three peptides [25 μM (P)PAP-A3 and 50 μM IMY25 and FLK22] against increasing concentrations of alginate (Figure 20).

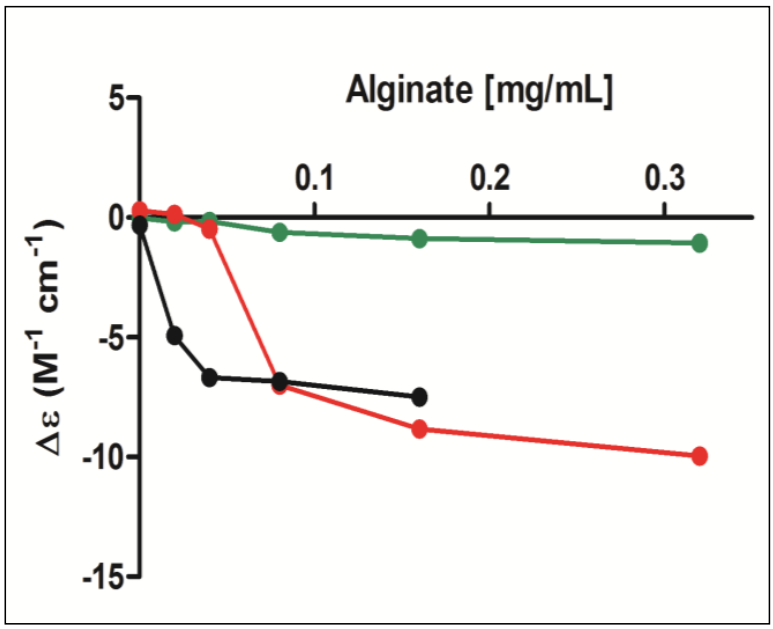

Figure 20. Pepsinogen A3 derived peptides interaction with alginate. *Y axis reports molar absorption per residue (Δε. M^{-1} cm^{-1}) at 196nm of (P)IMY25 (green line), (P)FLK22 (red line) and (P)PAP-A3 (black line) as function of alginate concentration reported on the X axis. Spectra were obtained in 10 mM buffer phosphate.*

Alginate induced a remarkable change in the spectra of three peptides, confirming the ability of alginate to cause their unfolding from conformations rich in beta structure and turns to random coil and/or a polyproline helix. Moreover, there were significant differences among the three peptides, and their binding with the alginate as evidenced by the plot in Figure 20. The absolute $\Delta\varepsilon_{196nm}$ values of (P)PAP-A3 and (P)FLK22 were very high suggesting a

probable structure change following the interaction with the Alginate. The $\Delta\varepsilon_{196nm}$ values found for (P)IMY25 were considerably lower than those of the two other peptides, suggesting either a weaker binding to the polysaccharide or a lack of significant structural change upon binding.

4.2.9 Peptide cytotoxicity and hemolytic activity

The pharmacological use of any potential drug cannot overlook an accurate investigation of their toxicity towards human cells. The toxicity of AMPs towards eukaryotic cell lines is variable and some AMPs show toxicity already at slightly higher concentrations than their MIC values. The toxicity of the pepsinogen A-derived AMPs for cells in culture is not particularly relevant, because the mucous layer, secreted by the epithelial cells, protects gastric and duodenal mucosa from the aggression of chloride acid and of the gastric proteases [84]. This mucous layer can also protect cells from the peptides.
To verify if PAP-A3, (P)IMY25 and (P)FLK22 can be considered promising candidates for the development of antimicrobial agents, their hemolytic activity and toxicity against HaCaT cells, a human epithelial cell line, were evaluated. As shown in Figure 21, (P)PAP-A3, at concentrations up to 40 μM and for incubation times up to 72 h, had negligible effects on murine erythrocytes or HaCaT cells, similar results were obtained for (P)IMY25 and (P)FLK22 treatments (data not shown).

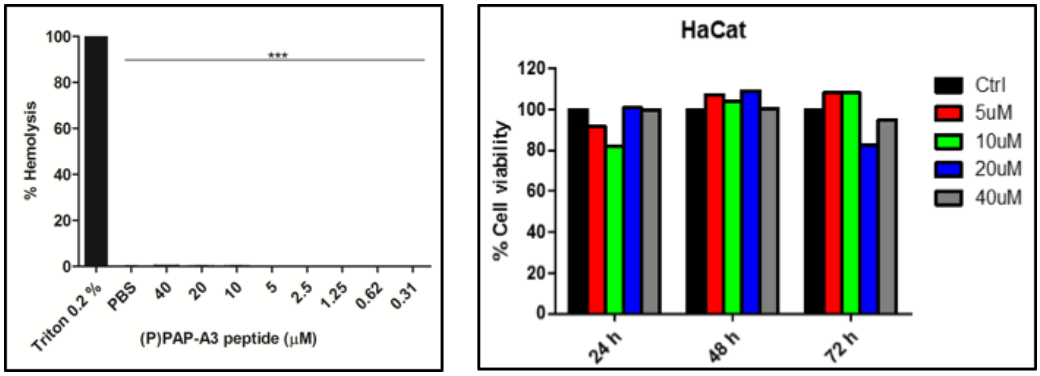

Figure 21. Cytotoxicity of recombinant peptide (P)PAP-A3 for eukaryotic cells. ***A)*** *Peptide lytic effects were evaluated on murine erythrocyte upon incubation for 1 h at 37 °C with increasing concentrations of (P)PAP-A3 or Triton X-100 0.2 % used as positive control. Hemolysis was normalized to the* A_{450} *of Triton X-100-treated erythrocyte samples and reported as a percentage.* ***B)*** *HaCaT cell viability (as percentage of total HaCaT cells) upon peptide (P)PAP-A3 treatment was determined by an MTT assay. HaCaT cells were incubated for up to 72 h with increasing concentrations of peptide or water (control). Reported data points are the average of three biological replicates. SD values were lower than 0.1 for each data point.*

4.3 Lignan-like compounds

4.3.1 Synthesis of tested furans

Starting arylfurans 1-3 (Figure 22) were prepared by $NaBH_4$ reduction of the corresponding dimethyl 2-arylfuran-3,4-dicarboxylates by co-workers of Chemical Sciences Department, University of Naples Federico II [85] as described previously [86].

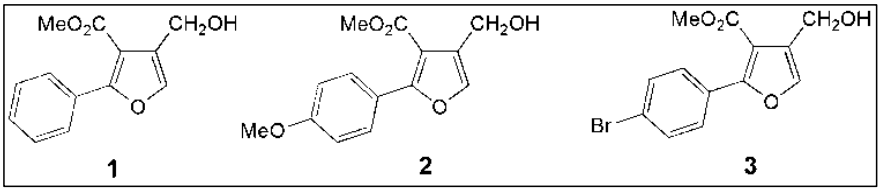

Figure 22. *Structures of arylfurans 1-3*

Compounds 1-3 were used as alkylating reagents in a variant of Friedel-Crafts reaction with arenes (anisole, 1,2-dimethoxybenzene and phenol). This reaction uses a combination of Tf_2O and Ph_3PO and represents an environmentally conscious methodology for lignan-like arylbenzylfurans 4-14 (Figure 23).

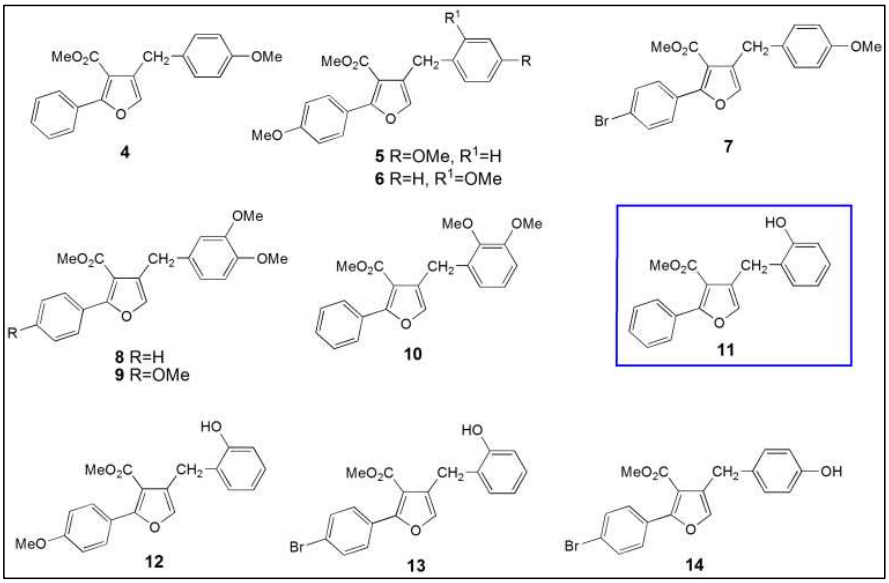

Figure 23. *Structures of lignan-like arylbenzylfurans 4-14.*

4.3.2 Antimicrobial activity of compounds against Gram-positive and Gram-negative bacteria.

A dilution assay was performed against a variety of representative pathogenic Gram-negative and Gram-positive bacterial strains, including *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. epidermidis* ATCC35984, *S. aureus* ATCC12600, to assess the potential antimicrobial activity of the fourteen synthetized compounds: aryl furans 1-3 and lignan-like furans 4-11, hereafter denoted with numbers from 1 to 14. The antibacterial activity was evaluated and expressed as the lowest drug concentration yielding decrease in absorbance compared with the untreated control (MIC). As shown in Table 5, Compound 11 was the only one showing antimicrobial activity against *S. aureus* and *S. epidermidis* with a MIC value of 4 μg/mL. A MIC value of 64 μg/mL was observed with compound 13 against both tested Gram-positive bacteria. For compounds 9 and 12 antimicrobial effects were observed at a MIC value of 128 μg/mL. Finally, none of the tested compounds showed any effect on *P. aeruginosa* and *E. coli* growth.

Compounds	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*S. epidermidis* ATCC 35984	*S. aureus* ATCC 12600
1	>128	>128	>128	>128
2	>128	>128	>128	>128
3	>128	>128	>128	>128
4	>128	>128	>128	>128
5	>128	>128	>128	>128
6	>128	>128	>128	>128
7	>128	>128	>128	>128
8	>128	>128	>128	>128
9	>128	>128	128	128
10	>128	>128	>128	>128
11	>128	>128	4	4
12	>128	>128	128	128
13	>128	>128	64	64
14	>128	>128	>128	>128

Table 5. In vitro antibacterial activity of tested compounds. *MIC values for compounds 1-14 against E. coli ATCC25922, P. aeruginosa ATCC27853, S. epidermidis ATCC35984, S. aureus ATCC12600. Values are expressed in μg/mL.*

The antimicrobial activity of Compound 11 was further investigated against Methicillin-resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE) and showed the same effects at the same concentration reported above (4 μg/mL), (Table 6). The microorganisms were not

susceptible to the concentration of DMSO used to dilute each compound (negative control).

Compounds	*S. epidermidis* MRSE	*S. aureus* MRSA
11	4	4

Table 6. In vitro antibacterial activity of tested compounds. *MIC values (μg/mL) for Compound 11 against clinical multi-drug resistant strains.*

4.3.3 Effect of Compound 11 and 13 on cells viability

The eventual cytotoxic effects of Compound 11 and Compound 13 on HaCaT cells was tested at the concentration of 2, 4, 8, 16, 32, 64 and 128 μg/mL. As demonstrated by MTT assay (Figure 24), Compound 11 didn't show cytotoxic effects at none of the tested concentrations. In contrast, compound 13 induced a 50% reduction of HaCaT cells viability already at 32 μg/mL. For this reason, the subsequent experiments were performed only using Compound 11.

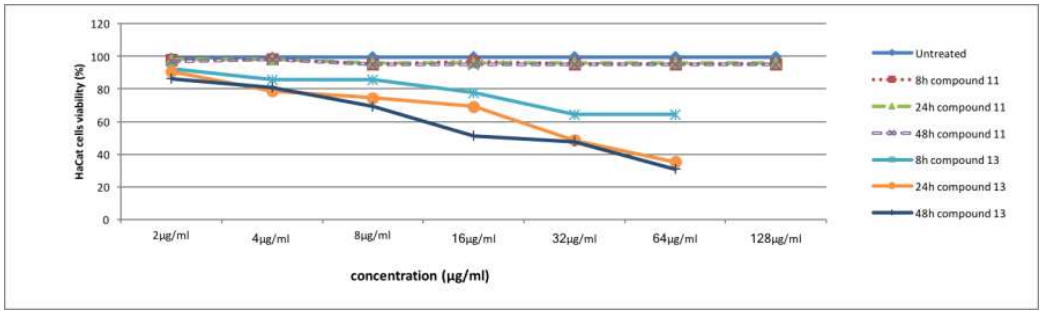

Figure 24. Time course assessment of treatment with different concentrations of Compound 11 on HaCaT cells viability assessed by MTT assay. *HaCaT cells were treated (or untreated) with various concentrations of Compound 11 (ranging from 2 μg/mL to 128 μg/mL) and viability of cells recorded at 8, 24 and 48 h.*

4.3.4 Time kill assay

Table 7 and Figure 25 show the results of a time kill assay performed by plating onto TSA agar of untreated cells and treated cells using Compound 11 at MIC concentration (4 μg/mL). The results on viability, measured as delta $\log_{10}$ CFU/mL of viable colonies, indicates that Compound 11 inhibits bacterial growth already at 2 h,

with a percentage of CFU reduction of about 40%, compared to untreated bacteria (5.39 and 4.95 log_{10} CFU/mL of treated *S. aureus* and *S. epidermidis* versus 8.77 and 7.34 log_{10} CFU/mL of respectively untreated cells). The inhibitory effect of Compound 11 was clearly visible up to 6 h. After 24 h of incubation, an increase of cell growth was observed, confirming a bacteriostatic effect on *S. aureus* and *S. epidermidis* growth.

	Log_{10} CFU/mL				
	0h	2h	4h	6h	24h
***S. aureus* untreated**	5.51	8.77	8.79	9.09	9.36
***S. aureus* + compound 11**	5.45	5.39	5.04	4.81	8.08
***S. epidermidis* untreated**	5.11	7.34	7.89	8.54	8.99
***S. epidermidis* + compound 11**	4.89	4.95	4.79	4.91	7.67

Table 7. In vitro time-kill assessment of Compound 11 against bacterial strains. *S. aureus ATCC12600 and S. epidermidis ATCC35984 were exposed at the concentration of MIC (4 µg/mL) for various durations (2h, 4h, 6h and 24 h). The inhibitory effect on bacterial growth was assessed by measuring the number of CFU obtained after the treatment. Experiments were performed in triplicates.*

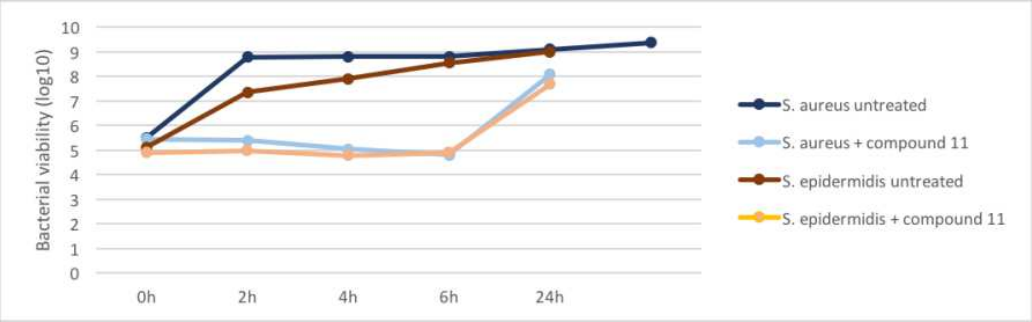

Figure 25. In vitro time-kill assessment of Compound 11 against bacterial strains. *Time-kill curves. The graph is plotted as the logarithm of the number of remaining viable cells (log_{10} CFU/mL) against time.*

4.3.5 Effect of Compound 11 on microbial biofilm formation

Further experiments were focused on the effects of Compound 11 on *S. aureus* and *S. epidermidis* biofilm formation. Compound 11 was tested at sub-MIC concentrations ranging from 0,5 µg/mL to 2 µg/mL for 24 h. Quantification of crystal violet staining by measurement of OD_{595} showed the ineffectiveness of Compound 11 on *S. aureus* and *S. epidermidis* biofilm formation (Figure 26). In fact, the tested

compound was not able to alter biofilm formation process in any of the tested concentration.

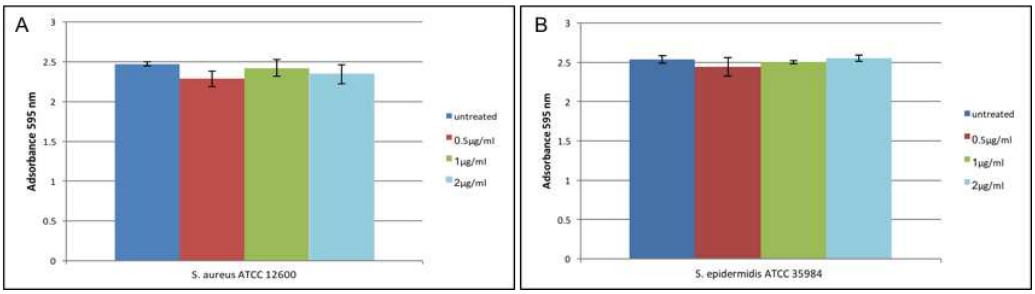

Figure 26. Effects of different sub-MIC concentrations of Compound 11 on S. aureus and S. epidermidis biofilm formation. A) *and* ***B)*** *Optical density of S. aureus ATCC12600 and S. epidermidis ATCC35984 biofilm, respectively, assessed by crystal violet assay. Experiments were performed in triplicate in three independent experiments.*

4.3.6 Effect of Compound 11 against mature biofilms

S. aureus and S. epidermidis can adhere to the medical devices and produce biofilms, thus increasing their resistance to antimicrobial agents. It was thus of interests to evaluate the activity of Compound 11 on preformed biofilms. One-day-old-biofilms, formed on a 96-well plate, were incubated with Compound 11 at a concentration of 8xMIC (32 µg/mL), 4xMIC (16 µg/mL) and 2xMIC (8 µg/mL) for 24 h. The obtained results revealed that Compound 11 had no effect on the biomass of the treated biofilm but yielded a significant decrease of biofilm viability compared to the corresponding untreated control (Figure 27). In particular, the compound exhibits notable anti-biofilm activity against *S. aureus* and *S. epidermidis* preformed biofilms at very low concentrations. The effect was already appreciable at the value of 2xMIC (8 µg/mL). The minimal concentrations which eradicated the 90% ($MBEC_{90}$) of *S. aureus* and *S. epidermidis* preformed biofilm were 32 µg/mL and 8 µg/mL, respectively.

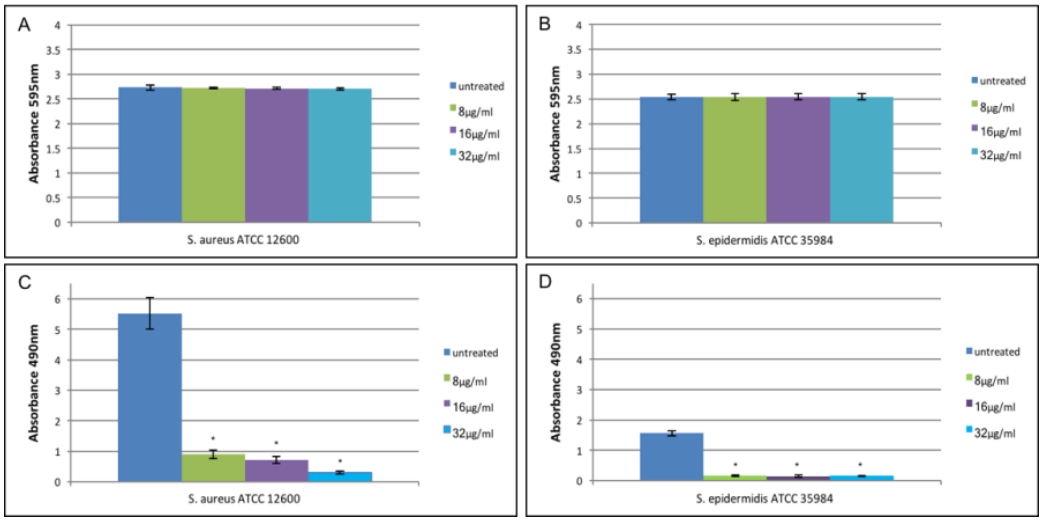

Figure 27. Effect of different concentrations of Compound 11 on one-day-old biofilm of S. aureus ATCC12600 and S. epidermidis ATCC35984*. Panels* ***A)*** *and* ***B)*** *report biofilm biomass of S. aureus and S. epidermidis assessed by crystal violet; panels* ***C)*** *and* ***D)*** *report biofilm viability obtained by XTT reduction assay. Bar colours represent different tested concentrations. Data are presented as the mean ± one s.d. from three independent experiments. * indicates significant (T-student test* $p < 0.05$*) difference compared with untreated control. Experiments were performed in triplicate in three independent experiments.*

To further corroborate the results obtained so far, the anti-biofilm property of Compound 11 was investigated by CLSM, using BacLight LIVE/DEAD staining kit. As showed in Figure 28 the visual impact of the effect of Compound 11 on mature biofilms is clear. Compared to control cells, *S. aureus* and *S. epidermidis* biofilms vitality were strongly reduced after the treatment with Compound 11 at 8xMIC (32 µg/mL). Dead cells (marked in red) were abundantly present in biofilm treated with compound 11, whereas alive cells (marked in green) were the majority in control sample.

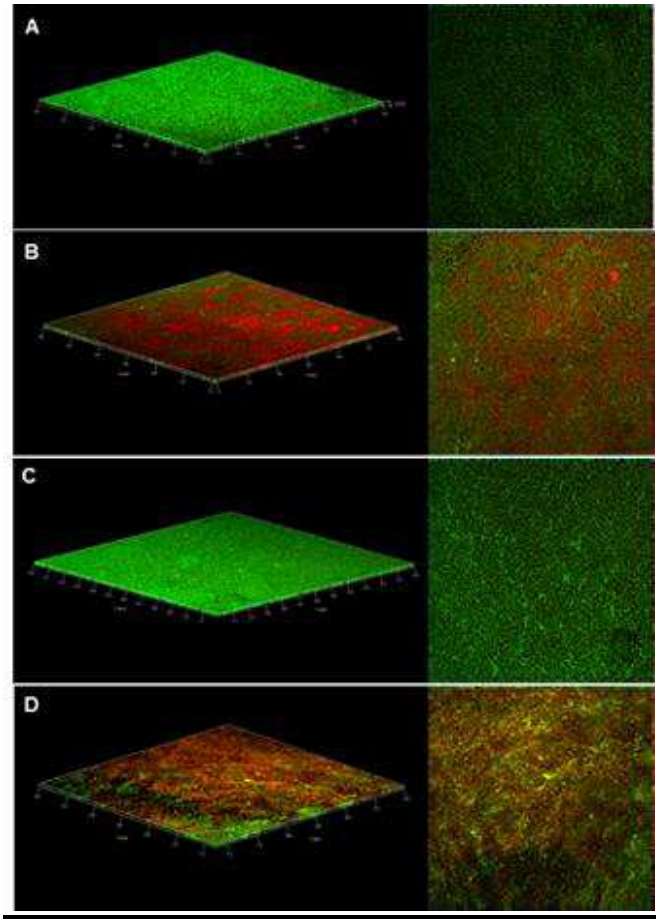

Figure 28. Effect of Compound 11 against S. aureus ATCC12600 and S. epidermidis ATCC35984 preformed biofilm. *Confocal laser scanner microscopy micrographs of S. aureus biofilm untreated* ***A)****, S. aureus biofilm treated with Compound 11 at 8xMIC (32 μg/mL)* ***B)****, S. epidermidis biofilm untreated* ***C)****, S. epidermidis biofilm treated with Compound 11 at 8xMIC (32 μg/mL)* ***D)****. Left panels contain three-dimensional images, right panels contain orthogonal images of biofilm. Green and red fluorescence is associated with live and dead cells, respectively.*

4.4 Abietic acid

4.4.1 In vitro assay of antimicrobial activity

Antimicrobial activity of Abietic acid was evaluated in terms of bacterial growth inhibition through minimum inhibiting concentration (MIC) by microdilution method. Abietic acid was tested against different *S. pseudintermedius* strains, including strains with an important chemo-sensibility spectrum, such as Methicillin-resistant *S. pseudintermedius* (MRSP). This compound showed a considerable bacteriostatic activity, with significant MIC value at 32 μg/mL and 8 μg/mL against MRPS and MSSP strains respectively (Table 8). The minimal bactericidal concentration was obtained after dilutions and plating on TSA agar. Abietic acid showed a bactericidal effect at 64 μg/mL (2xMIC) and 32 μg/mL (4xMIC) against MSSP and MRSP strains respectively.

Strains	MIC (μg/ml)	MBC (μg/ml)	Methicillin (μg/ml)
MSSP 1	8	32	<0,25
MSSP 2	8	32	<0,25
MRSP 1	32	64	10
MRSP 2	32	64	10

Table 8. MIC and MBC values of Abietic acid on selected bacterial strains. *Each experiment is the result of three independent experiments each one performed in triplicate.*

4.4.2 Time Kill Assay

Time kill assay was performed on MSSP and MRSP strains at the MIC concentrations of 8 μg/mL and 32 μg/mL respectively. Table 9 shows the $\log_{10}$ CFU/mL concentration of living cells. The MIC concentration of Abietic acid can increase the reduction rate of total viable colonies compared to untreated control for both strains. Abietic acid was able to inhibit bacterial growth already at 1 h, with a percentage of CFU reduction of 60%, and 30% compared to untreated bacteria for MSSP and MRSP, respectively. Figure 29 clearly indicates how the highest inhibition activity of Abietic acid on both bacterial strains can be obtained after 6 h exposure, where a reduction of 90% and 75% was respectively observed for MSSP and MRSP.

After 24 h of incubation, an increase of cell growth was observed, confirming a bacteriostatic effect of Abietic acid on both *S. pseudintermedius* strains.

		Log_{10} CFU/mL				
	0h	1h	2h	4h	6h	24h
***S. pseudointermedius* MSSP 1 untreated**	5.60	5.84	5.90	5.97	7.30	9.77
***S. pseudointermedius* MSSP 1+ abietic acid**	5.50	2.20	1.60	1.30	1	5.30
***S. pseudointermedius* MRSP 1 untreated**	5.30	5.47	5.74	5.81	6.91	8.36
***S. pseudointermedius* MRSP 1+ abietic acid**	5.40	3.77	3.38	2.30	1.77	5.32

Table 9. In vitro time-kill assay of Abietic acid against bacterial strains. *Clinical strains were exposed at the MIC concentration (8 µg/mL and 32 µg/mL) at different times (2 h, 4 h, 6 h and 24 h). The inhibitory effect on bacterial growth was assessed by measuring the number of CFU obtained after the treatment. Experiments were performed in triplicates.*

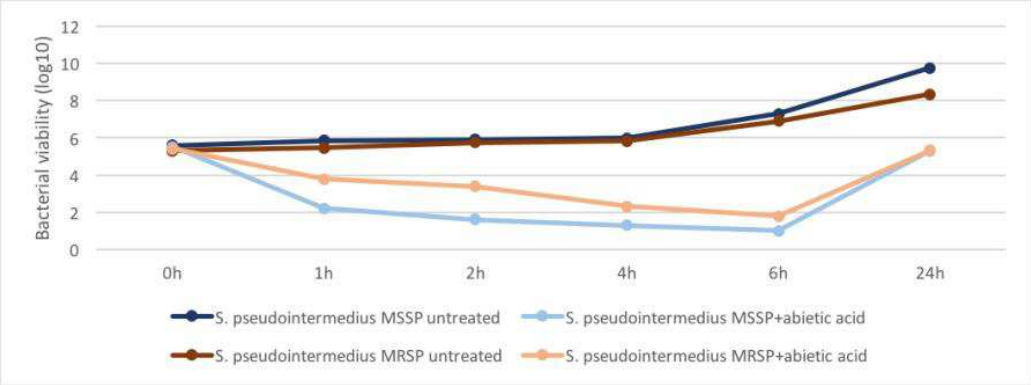

Figure 29 In vitro time-kill assay of Abietic acid against bacterial strains. *The graph is plotted as the logarithm of the number of remaining viable cells (log_{10} CFU/mL) against time.*

4.4.3 Synergistic study

To improve the antibiotic efficiency as well as to reduce the antibiotic dose, the antibacterial activity of combinations of Abietic acid and Methicillin were tested on MRSP by means of checkerboard assay. The MIC of Abietic acid was determined to be 32 µg/mL while the MIC of Methicillin was 10 µg/mL. In this assay antibiotic and Abietic acid were used alone and in combination against MRSP planktonic cells. The Abietic acid and Methicillin concentrations decreased along the rows and column from 32 µg/mL to 0 µg/mL and from 10 µg/mL to 0 µg/mL, respectively (Figure 30).

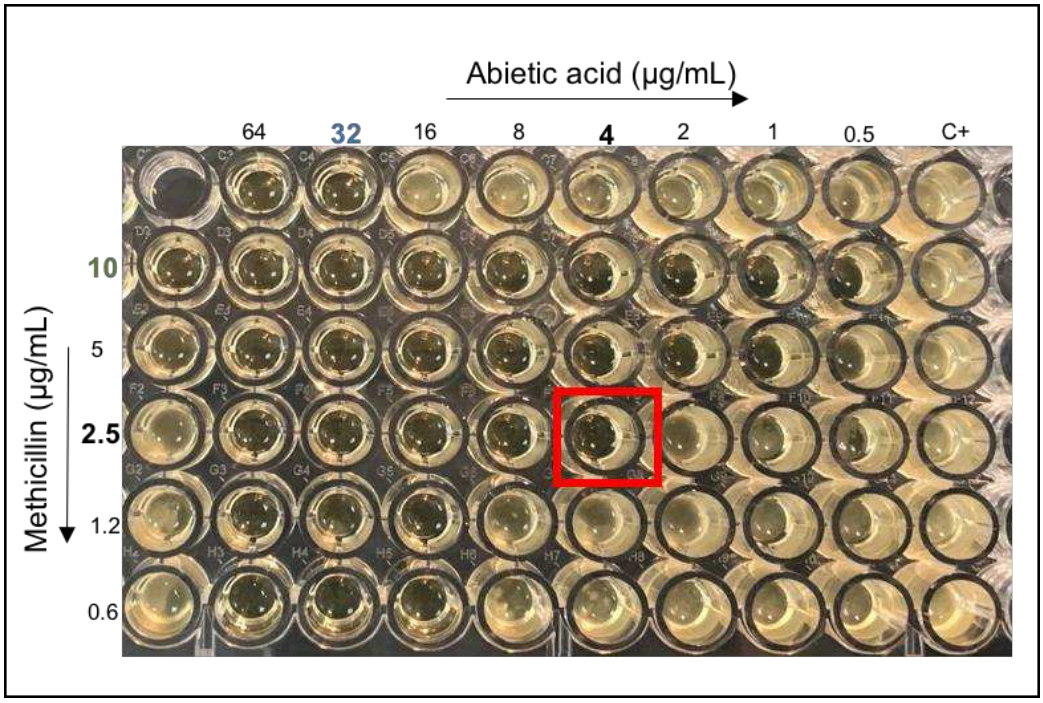

Figure 30. Checkerboard assay used to test the interaction between Abietic acid and Methicillin. *Turbidity (lighter wells) represents bacterial growth while absence of turbidity (darker wells) represents inhibition of bacterial growth. MIC value of Methicillin alone (10 µg/mL) is reported in green, while MIC value of Abietic acid alone (32 µg/mL) is reported in blue. MICs combination are reported in bold black for both compounds. The red square highlights the best combination of Abietic acid and Methicillin.*

The highest synergistic interaction against MRSP was obtained in the wells without turbidity with the best combination of values of 4 µg/mL concentration (1/8 MIC) of Abietic acid and Methicillin at 2,5 µg/mL (1/4 MIC). The calculation of FIC index, equal to 0,375, confirmed the synergistic effect of Abietic acid and Methicillin.

4.4.4 Mec genes expression

The mechanism of Methicillin resistance is usually conferred by the acquisition of a gene encoding PBP2a, a penicillin-binding protein characterized by a considerably low affinity for β-lactams. This latter allows cell-wall biosynthesis (the target of β-lactams) to continue even in the presence of inhibitory concentrations of antibiotic (Figure 31).

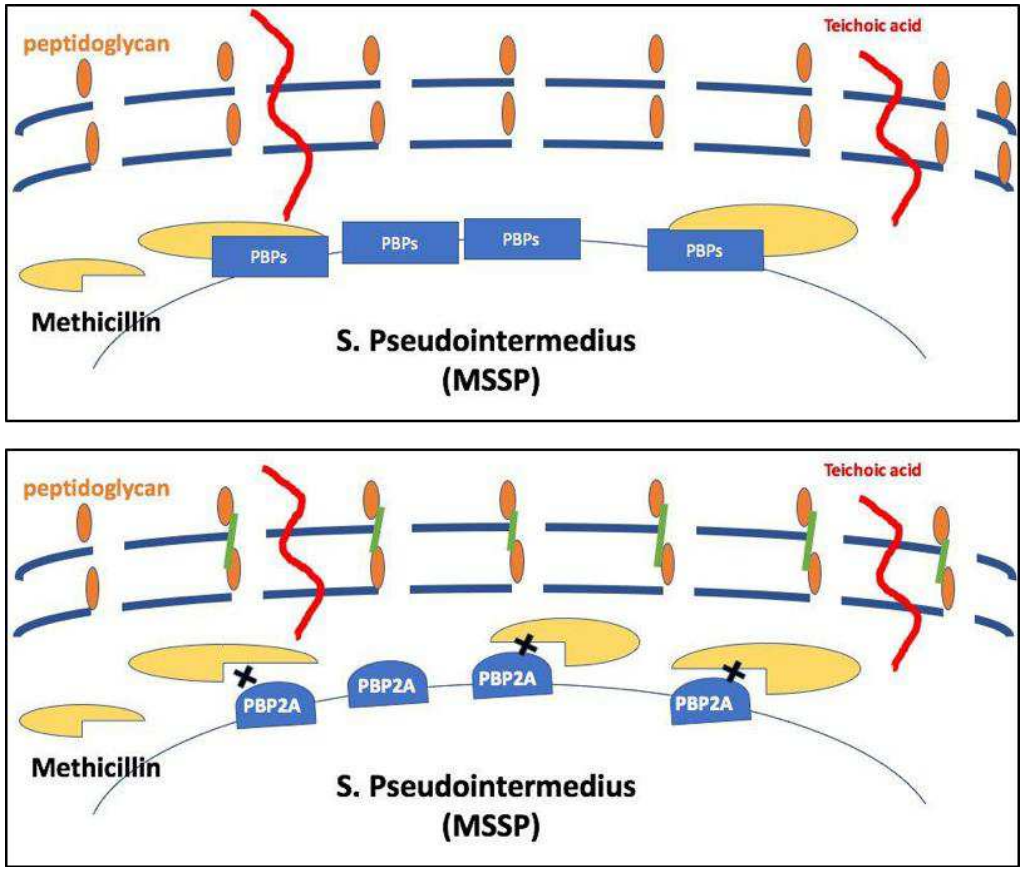

Figure 31: Activity models of methicillin on MSSP and MRSP. *Top panel: Action of Methicillin against MSSP. Methicillin inhibits peptidoglycan synthesis by binding to PBPs. Bottom panel: mechanism of methicillin resistance in MRSP. S. pseudointermedius produce unique PBP2A, consequently methicillin cannot bind to the target PBP and peptidoglycans synthesis continues despite the Methicillin presence.*

This modified penicillin-binding protein is encoded by the *mecA* gene, which is regulated by mecI-mecR systems. MecR1 is a transmembrane spanning and signal transducing protein. The acylation of MecR1, consequent to an external interaction with beta-lactam antibiotics, is followed by auto-proteolytic cleavage on the cytoplasmic side of the cell membrane. The separated intracellular portion of MecR1 travels to the bacterial chromosome and removes its cognate repressor MecI via proteolysis. Once the repressor dissociates from its promoter region binding site, transcription of the *mecA and mecR1/mecI/mecR2* genes begins. MecR2 disturbs the binding of the repressor MecI to the *mecA* promoter, which leads to its proteolytic inactivation, sustaining *mecA* induction. MecR1 result to be not activated in the absence of β-lactams, in this case a steady state is established instead, with stable MecI-dimers that are bound to

the *mecA* promoter and residual copies of MecR1 that are bound to the cell membrane [87] (Figure 32).

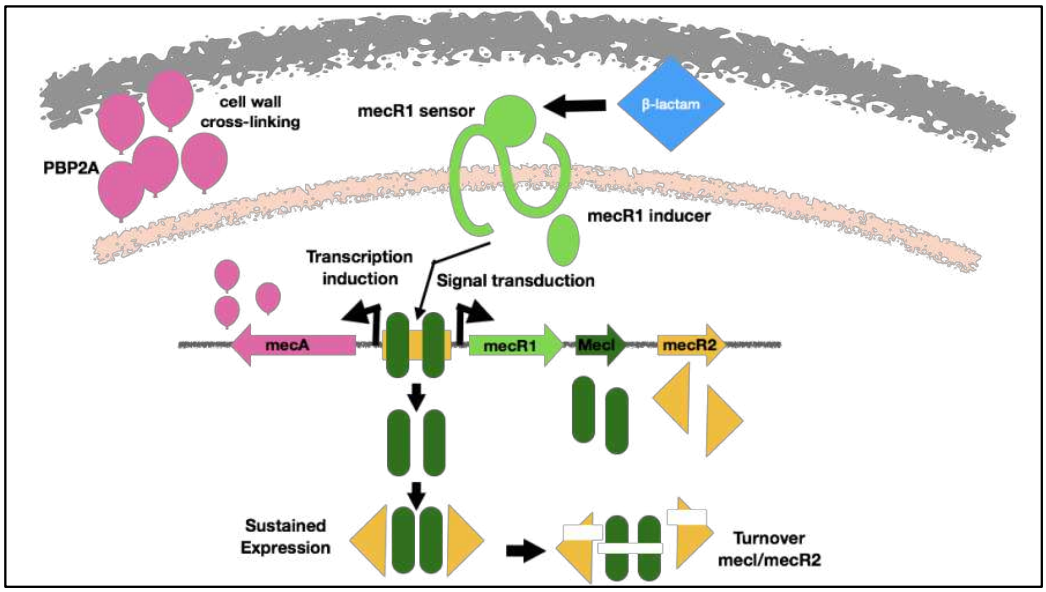

Figure 32. Model of mecA induction by MecR1-MecI-MecR2 system. *In the presence of a β-lactam antibiotic, MecR1 is activated and rapidly induces the expression of mecA and mecR1-mecI-mecR2 genes.*

On the basis of this, real-time PCR was performed to investigate if Abietic acid, alone or in combination with Methicillin, influences the expression of *mec* genes. Quantification data for all genes were normalized to the reference gene for rRNA16s. In Figure 33 is reported the relative expression of MRSP genes after 30 min and 1 h treatment with Abietic acid alone at the MIC concentration (32 µg/mL). Results show how the Abietic acid induces a general downregulation of genes from *mec* operon in a time dependent manner (Figure 33).

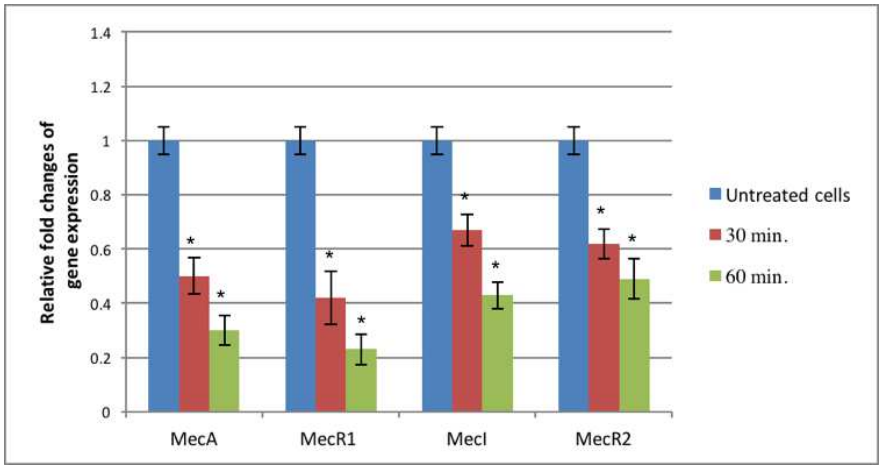

Figure 33. Relative expression of mec operon genes. *MRSP was treated with MIC concentrations of Abietic acid from for 30 min and 1 h. Transcript levels were monitored by real-time PCR. Using the* $2^{-\Delta\Delta Ct}$ *method. The data are presented as the fold change in gene expression normalized to an endogenous reference gene (rRNA 16s) and relative to the untreated control (value 1). Bars represent the mean ± SD for three independent experiments. * indicates statistically significant difference (T-student test p < 0.05).*

Finally, the expression of *mecA* gene in MRSP was examined after treatment with Abietic acid and Methicillin in combination at the concentration of 1/8 MIC (4 µg/mL) and 1/4MIC (2,5 µg/mL) respectively and at different times (30 min and 60 min). Methicillin alone upregulated the expression of *mec*A gene. The addition of Abietic acid to methicillin not only reduced the expression of *mec*A gene in comparison with untreated control, but also inhibited the induction of expression compared to cells treated with only Methicillin (Figure 34).

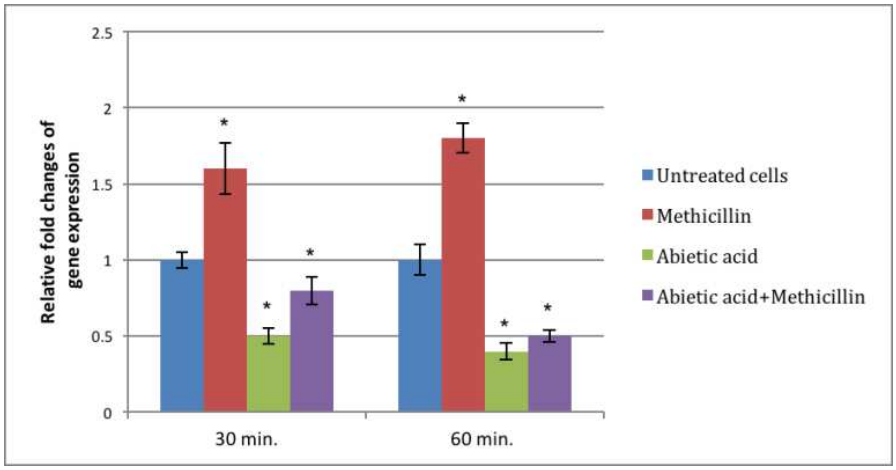

Figure 34. Relative expression of mecA gene in MRSP at different exposure time. *Blue bars: untreated cells; red bars: treated with only Methicillin; green bars: treated with Abietic acid alone; purple bars: treated with Abietic acid and Methicillin. Data are presented as fold change in gene expression normalized to an endogenous reference gene (rRNA16s) and relative to the untreated control. Values represent the mean ± 1 s.d. for three independent experiments. * indicates statistically significant difference (T-student test $p < 0.05$).*

4.4.5 Anti-biofilm activity

The ability of Abietic acid to inhibit the formation of biofilms was investigated by crystal violet assay, evaluating the percentage of total biofilm biomass after 24 h of treatment. The sub-MIC tested concentrations ranged from 0,5 µg/mL (1/64 MIC) to 2 µg/mL (1/16 MIC) because concentration higher then 2 µg/mL were observed to only slightly inhibit bacterial growth. Abietic acid showed a good inhibiting activity for biofilm of MSSP. Also, it showed a considerable ability in reducing the biomass of MRSP biofilms, causing a 70% inhibition at the concentration of 2 µg/mL (Figure 35). The inhibition of biofilm formation of MSSP was dose dependent and it resulted to be statistically different from the control for each tested concentration. On the other hand, Abietic acid was not able to inhibit the biofilm formation of MRSP at any tested concentration.

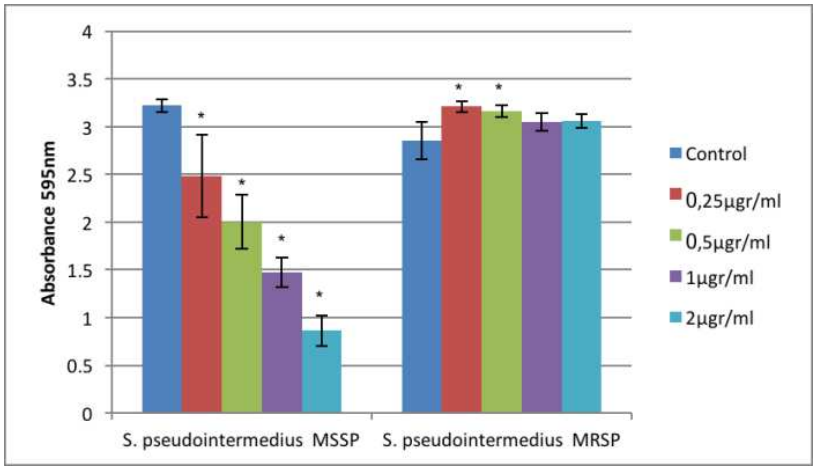

Figure 35. Activity of the Abietic acid on S. pseudintermedius biofilm formation*. The test was performed at three times in triplicate. Values represent the mean ± 1 s.d. for three independent experiments. * indicates statistically significant difference (T-student test p < 0.05).*

The late stage of preformed biofilm could be more difficult to treat than the early- and mid-stages tested above. Therefore, the efficiency of Abietic acid against one-day-old biofilm was assessed by crystal violet staining and XTT assay, after 24 h incubation with the compound. Cristal violet assay showed that Abietic acid did not exert a considerable anti-biofilm activity at concentrations up to 160 µg/ml. In fact, at the maximum tested concentration, it was able to reduce only the 30% and the 50% of the biomass of MRSP and MSSP respectively (figure 36 - A). Regard biofilm vitality, a concentration of 10 µg/mL of Abietic acid, (corresponding to the value just above MIC of MSSP and 1/3 MIC of MRSP) was already sufficient to reduce the biofilm vitality by 50% and 30% of MRSP and MSSP respectively. Moreover, Abietic acid caused the decrease of approximately 80% of living cells of treated biofilms in both strains as compared to untreated biofilms with 20 µg/mL and 40 µg/mL doses for MRSP and MSSP respectively (Figure 36 - B). Finally, the impact of Abietic acid on the mature biofilm of MSSP and MRSP was observed by confocal microscopy at the doses 2xMIC (20 µg/mL and 40 µg/mL). Images of treated biofilms of both strains, showed a deep and compact red signal from the top layers to innermost, representing cells within biofilm that were killed by Abietic acid (Figure 36 - C).

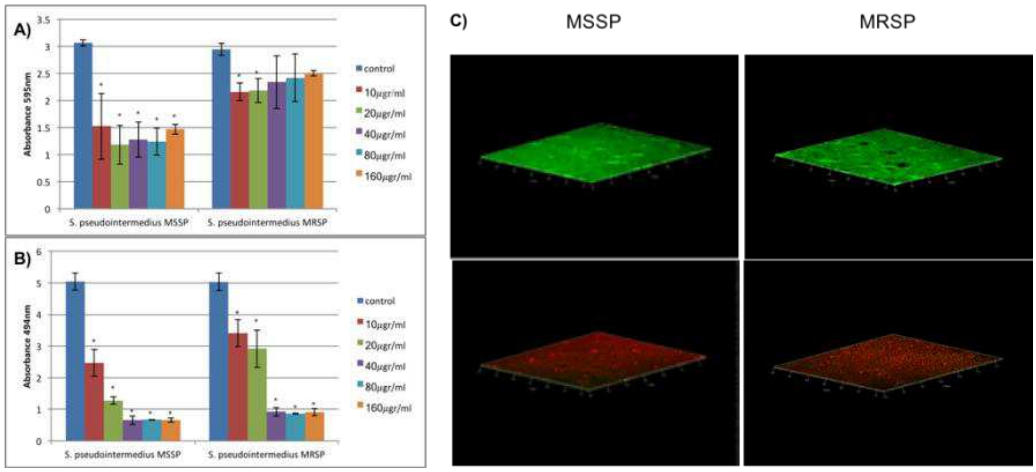

Figure 36. Effect of Abietic acid on one-day-old biofilms of S. pseudintermedius strains. *Standardized overnight cultures of S. pseudintermedius strains were grown in 96 polystyrene multi-wells overnight at 37 °C to form mature biofilms. Abietic acid was added to the wells at different concentrations (40 µg/mL, 80 µg/mL and 160 µg/mL), plates were incubated at 37 °C overnight to allow to the Abietic acid to act on the mature biofilms.* ***A)-B)*** *Biofilm biomass obtained by crystal violet staining and Biofilm vitality obtained by XTT assay. Bars represent the average values ± 1 s.d. from three independent experiments. * represents significant difference compared with untreated control (T-student test* $p < 0.05$*)* ***C)*** *CLSM micrographs. Upper panels show 48h-old untreated biofilm, bottom panels contain Abietic acid treated samples. Abietic acid was added to 24h-old biofilm of MSSP and MRSP at 40 µg/mL. After 24 h of incubation chambers were washed and images were acquired.*

5. DISCUSSION

Discussion

Candida species are the most common opportunistic fungal pathogens worldwide. Due to the growing risk of observing the insurgence of resistance to the most common antifungal drugs in this species, novel agents, active not only against *Candida* but also against biofilm-grown *Candida,* are strongly required. In this regard, antimicrobial peptides seem to be very promising candidates [88].
In this work, the antifungal and anti-biofilm activity of a fragment of transcriptional factor, isolated from *Sulfolobus islandicus*, was investigated in vitro against clinical isolates of *C. albicans* and other non-*albicans* species. VLL-28 exhibited good fungicidal activity against almost of planktonic *Candida* spp. isolates. This finding is in line with previous studies were the authors observed the ability of VLL-28 to kill bacteria and fungi growing in planktonic form [66], including a collection of *Candida* species isolated from clinical samples. Nowadays the most used antifungal agents have a specific mechanism of action such as the Azoles, that act by interfering with the biosynthesis of membrane ergosterol, or the Echinocandins that work by blocking the synthesis of membrane 1,3 beta-glucan and Polyenes, which induce the membrane pore formation by binding with the ergosterol. Instead VLL-28, like other amphipathic AMPs, appear to exert its antimicrobial effects by binding the cellular membrane and then increasing its permeability. This last mechanism is faster and less specific than the Azole mechanism. For this reason, the development of microbial resistance to AMPs is generally less likely to arise if compared to classic antifungal agents. However, concerning the *non-albicans Candida* spp. isolates, VLL-28 displayed an overall reduced activity towards the strains of *C. glabrata,* with higher MIC and MBC values (50 μM and 100 μM respectively). This observation is in line with published reports on poor susceptibility of *C. glabrata* to various cationic AMPs [89] and may reflect the unique and distinctive features of the cell wall of this pathogen [90]. Conventional antifungal drugs, such as Amphotericin B or Fluconazole, result to be almost always effective against *C. albicans* in its planktonic state but not against its biofilm formations [91-92]. Several factors have been suggested to be involved in the tolerance of biofilm to these antimicrobial drugs, including expression of resistance genes, such as those coding for efflux pumps [93], and the major features of extracellular matrix components, which prevent antifungals from reaching their targets [94]. Thus, an extremely limited drug arsenal is available to treat biofilm-related

Candida infections. VLL-28 effectively reduced the cells adhesion to abiotic surface of all tested strains except that of *C. krusei*. The concentration of ½ MIC inhibited the 80% of cells adhesion for almost tested strains. The ability of this peptide to prevent biofilm formation is particularly important for medical devices implantations. In fact, microbial colonization of indwelling medical implants with subsequent biofilm formation can lead to severe complications associated with increased morbidity and mortality, such as bloodstream infections and systemic inflammation [95]. Thus, it is very important to prevent microbial adhesion and biofilm formation on the surfaces of these devices. The ability to target biofilms formed by non-*albicans Candida* species is currently a hot topic, since infection by these species is becoming increasingly prevalent [96]. In this view, the capability of VLL-28 to kill mature biofilms formed not only by *C. albicans* but also by *C. glabrata* and *C. parapsilosis* is a valuable result and deserves further investigation in the perspective of developing novel peptide-based antifungal agents. In the performed experiments VLL-28 showed the ability to reduce 80% of metabolic activity of mature biofilm of *C. albicans* stains and 50% of *C. parapsilosis* and *C. glabrata* strains, with concentrations only from 2 to 4-fold higher than those needed for the planktonic state. VLL-28 showed a different effect on the mature biofilms of *C. tropicalis* strains: it caused a considerably decrease of the biofilm biomass, whereas biofilm vitality only slightly decreased. Fattani et al. demonstrated that *C. tropicalis* have the ability to form compact biofilms due to the synthesis of large amounts of matrix [97]. For this reason, the interaction of VLL-28 with *C. tropicalis* biofilm matrix, could lead to the disaggregation of the biofilm and the delay the peptide diffusion within it, with the consequent impossibility to kill cells that are almost hidden by the enveloping matrix. These data constituted an important finding, because approximately 65% of all clinical infections by *Candida* are biofilm-associated [98]. *Candida* biofilm-related infections are difficult to treat, in fact minimal inhibitory concentrations for eradication of the biofilm-related infection are often dramatically more elevated than MICs in planktonic form. If we consider Azoles we have that *Candida* biofilms are up to 1000 times more resistant than the corresponding planktonic cells [99] while Echinocandin activity seems to be variable among *Candida* spp. [100]. These infections result to be very hard to treat with current drugs. These latter could be treated only with high antifungal doses in systemic therapy, but these doses can often be toxic for the human organism.

Human pepsinogen A3, A4 and A5 are the inactive forms of abundant gastric proteases pepsins A3, A4 and A5, which are synthesized and stored at neutral pH in the chief cells of the stomach. Once they are secreted, the acidic pH of lumen stomach, allows their conversion into active enzymes, essentially, by cleavage of the long N-terminal peptide (47 amino acids) known as pepsinogen activation peptide or prosegment [101]. Although the role of pepsin prosegment in the activation pathways have been well described [102-103], beyond safe storage and secretion of the protein, no other significant functions have been attributed to these peptides.

Here it was demonstrated that human pepsinogen A activation peptide has an important antimicrobial activity at physiological pH and concentrations. In the stomach, its bactericidal activity may complement the bacteriostatic/bactericidal activity of low pH values and the bactericidal activity of gastric aspartic proteases, helping to decrease the bacterial load of ingested food. On the other hand, when the food moves from the stomach to the duodenum, where bicarbonate secretion causes aspartic proteases to lose their catalytic activity and hence their bactericidal activity, the activation peptide can still exert its bactericidal role. From this point of view, the activation peptide could act as a substitute for lactoferrin-derived peptides in individuals whose diet does not include milk.

It has been also demonstrated that PAP-A3 and its fragments possess interesting anti-biofilm activities and that the determinants for this bactericidal and anti-biofilm activities are at least partially separated, being located at the N-terminus and the C-terminus, respectively. These finding also suggests that the partial cleavage of PAP-A3 into its two fragments could have a relevant physiological role, with the C-terminal retaining the disaggregating properties and the N-terminal portion retaining significant antimicrobial activity but with an increased ability to penetrate into biofilms. These findings could also help to explain an intriguing peculiarity of the pepsinogen physiology. It has long been known that a small but not negligible amount of pepsinogen is secreted in the bloodstream, filtered by the kidneys, and released unchanged in the urine [104]. This pepsinogen fraction, also known as “uropepsinogen”, when purified from urine and exposed to pH 2, undergoes autocatalytic processing to fully active pepsin [105-106]. However, in physiological conditions, autocatalytic activation in the urine is highly unlikely. Moreover, because pepsin is inactive at pH higher than 5, even postulating the existence in the urine of a protease able to release the activation

peptide, any role based on proteolytic activity can likely be excluded. On the other hand, the bactericidal and anti-biofilm activity of pepsinogen A-derived AMPs can be exerted at neutral pH, therefore, uropepsinogen could have a physiological function as an AMP precursor. Obviously, more targeted studies are required to verify this hypothesis.

Lignans can be used as a source for structural insight to guide molecular design of novel compounds. The structural motif, based upon furan-containing lignan-like compounds, was investigated here. The use of this heterocyclic system was motivated by the presence of the furan ring in a variety of natural products as well as by the easy preparation and reactivity of furans [107]. A furan derivative has been found having cytotoxic antibiotic activity and aryl furan moieties are present in a wide number of pharmaceuticals [108]. In this context, arylfurans, namely methyl 5-aryl-4-hydroxymethyl-3-carboxylates, were used as suitable scaffolds to obtain arylbenzylfurans with a typical lignan backbone.
Being common inhabitants of human skin and mucous surfaces, *S. aureus* and *S. epidermidis* bacteria are most frequently associated to infections of skin and soft tissue lesions resulting from trauma, burns or surgery, as well as to vascular catheter-related bloodstream infections. Biofilm formations play an important role in medical device infections, and in the recent years their relevance has been recognized in burns, grazes or surgical wounds infections as well. Several studies, performed by using *in vivo* animal wound models or *in vitro* keratinocyte cultures, have shown that biofilm formation delays the healing process and promotes chronicization of the wounds [109-110]. Since most antimicrobials cannot penetrate the extracellular matrix of biofilms, it is fundamental for new antimicrobials to be able to act both on the planktonic and sessile bacteria as well. In this study, the potential antimicrobial activity of synthetic lignans and aryl furans was tested against *S. aureus* and *S. epidermidis*. In the literature, there are few reports on the antimicrobial activity of lignans and its derivatives [111-112]. Among the fourteen tested compounds only compounds 11 and 13 showed considerable antimicrobial activity. The main difference in furan structures are the aryl substituents, generally typical of natural lignans as methoxyl and hydroxyl. The presence of hydroxyl on the aromatic moiety of compounds 11 and 13 appears to contribute to enhance their activity, as often found for natural lignans [58]. Anyhow, the position of hydroxyl appears to be important as pointed

out by the remarkable difference in activity of compounds 13 and 14. Compound 11 and compound 13 showed the best results against *S. aureus* and *S. epidermidis,* with a MIC value of 4 μg/ml and 64 μg/ml respectively, while both compounds resulted inefficacious against Gram-negative bacteria. This difference could be due to the particular structure of Gram-negative outer membrane that is resistant to most antimicrobial drugs. Thus, the cytotoxicity of Compound 11 and 13 was tested on an established human keratinocytes cell line (HaCaT). Compound 11 did not induce any modification of cell morphology or evident sign of cell death, instead compound 13 reduced HaCaT cells viability already at lower concentrations than MIC. For this reason, the subsequent studies were performed only for Compound 11. Interestingly, Compound 11 displayed its activity also against Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). This result is relevant because the frequency of *S. aureus* and *S. epidermidis* infections, the difficulty of their treatment and a rapid increase of Methicillin resistant strains represent a serious clinical concern. Compound 11 inhibited both *S. aureus* and *S. epidermidis* growth already at 2h, with a recover of cell growth after 24h of incubation. This result suggests the presence of a bacteriostatic effect rather than a bactericidal one. It is known that the first requirement for successful bacterial colonization is the attachment to abiotic surfaces of medical device or host tissues [113]. This process can be aspecific in the case of medical devices or take place through specific interactions with host receptors in host tissues. Since pathogenic microorganisms living in aggregation have enhanced virulence-related phenotypes, from a medical perspective, molecules acting in the initial phase of biofilm formation are of great interest because they can be used as a prophylactic measure. Moreover, if the molecules are effective also on preformed biofilm, they could be used to treat established biofilm infections as well. Consequently, it was investigated if Compound 11 was able to inhibit biofilm formation or disperse preformed biofilm.
The results reported here showed that Compound 11 was unable to inhibit biofilm formation while it was effective in reducing viability of one-day-old-biofilms at 2xMIC (8 μg/ml) for *S. epidermidis* and 8xMIC (32 μg/ml) for *S. aureus*. These results were confirmed by confocal laser scanning microscopy demonstrating that Compound 11 strongly affected *S. aureus* and *S. epidermidis* mature biofilm vitality.

Molecules that can destroy established biofilm may have a greater therapeutic potential; indeed, biofilm formation can take as little as a few hours and antimicrobial treatment often starts when biofilm has already been consolidated with most of currently used antimicrobials unable to penetrate the extracellular matrix. Moreover, the ability to penetrate and kill bacteria in biofilms is one of the most critical determinants for antimicrobial effectiveness on biofilms. Even after an antimicrobial treatment, some biofilms can still act as reservoirs for bacterial dissemination into systemic circulation and distant colonization of bacterial cells detached from mature aggregates [114,115]. Compound 11 seem to be able to penetrate the biofilm matrix and to interact with bacterial cells without any loss in its activity or binding processes while passing the EPS. This is a noteworthy feature since many antimicrobials are capable of disrupt the biofilm but not to eliminate biofilm bacteria, causing the release and the spread of live planktonic bacteria form the biofilm site [116].

In recent years, the high prevalence of Methicillin-resistant coagulase-positive staphylococci in veterinary world has become increasingly evident. [117]. Among Methicillin-resistant staphylococci (MRS), *Staphylococcus pseudintermedius* (MRSP) is a pathogen of great importance not only in veterinary but also in human medicine. In fact, the frequency and the risk factors to develop infections associated with colonization of MRSP in people in daily contact with animals is considerably increased [118]. Rodrigues et al. showed that the 60% of participants to their study were found to be colonized by at least one Methicillin-resistant (MR) staphylococci species. [119]. As a response to the imminent lack of effective antibiotics in veterinary field, and to the increase of zoonotic risk, here the antimicrobial properties of Abietic acid, a well-studied natural naphthenic acid, were investigated for this compound, alone and in combination with Methicillin, by evaluating its anti-biofilm properties against *S. pseudintermedius* strains. In literature, it was already reported that Abietic acid and its derived compounds possess an important antibacterial activity [62]. In the presented experiments Abietic acid showed a greater antimicrobial activity against *S. pseudintermedius* than *S. aureus* and *S. epidermidis*. In fact, Abietic acid had a MIC at 8 μg/ml and 32 μg/ml against *S. pseudintermedius* MSSP and MRSP, while Helfenstein et al. reported that 20 μg/ml and 8 μg/ml concentrations were able to inhibit the growth of *S. aureus*

and *S. epidermidis* by 44% respectively. In addition to having a great antibacterial activity, Abietic acid showed an important synergistic activity when used in combination with Methicillin. The Methicillin-resistance can be caused by different mechanisms, such as modified penicillin-binding protein (PBP). This last mechanism is the most diffused in Methicillin-resistant staphylococci species [120] where the *mecA* gene encodes a modified PBP, namely the PBP2a. *S. pseudintermedius* carrying *mecA* gene, is not only resistant to penicillins, cephalosporines and carbapenemes but often possess also resistance to macrolides, lincosamides and streptogramins. For this reason, the treatment options for infection by *Staphylococci* MR have become very limited. The results of synergistic assay here demonstrated that Abietic acid was able to increase Methicillin sensibility of MRSP through the reduction of the gene expression encoded by *mec* operon. Moreover, the effect of Abietic acid against *S. pseudintermedius* biofilm formation and maturation was valued. At much lower concentrations than MIC values Abietic acid is able to inhibits the 70% of biofilm formation, whereas at dose just above MIC, Abietic acid showed a drastic reduction of preformed biofilm vitality for both strains. Probably, due to its small structure, Abietic acid is able to penetrate the biofilm unlike large molecules such as aminoglycosides [121]. These findings might be of therapeutic interest because biofilms were considered one important virulence factors in several *Staphylococcus* spp. including *S. pseudintermedius*. *S. pseudintermedius* is able to form a biofilm ultra-structurally complex that is intrinsically resistant to antibiotics. [122]. In fact, in literature was reported that MRSP and MSSP associated with biofilms have significantly increased MIC to conventional antimicrobials compared to their planktonic counterparts [123]. The results discussed above suggest that Abietic acid can be considered a potential therapeutic agent especially when used in combinatorial antibiotic therapy.

6. CONCLUSIONS

Conclusions

VLL-28 is a peptide that is part of the primary structure of Stf76 protein, a transcription factor encoded by pSSVx, a hybrid plasmid–virus from the archaeon *Sulfolobus islandicus*. VLL-28 has a broad activity spectrum against *Candida* spp. by interacting with fungal membrane, it is effective in preventing fungal growth on polystyrene surfaces, and is able to kill *Candida* cells in a 48-h old biofilm. These results encourage further studies on this peptide in view of its possible development for antifungal applications.

Despite being generally considered a waste product, here it was demonstrated that recombinant activation peptide of human pepsinogen isoform A3 (PAP-A3) has a potent antimicrobial and bactericidal activity, at both pH 3 and pH 7, against a range of bacteria, including common intestinal bacteria and foodborne organisms that commonly infect the digestive tract. PAP-A3 may exert a protective function both in the stomach as well in the duodenum, where bicarbonate secretion neutralizes the acids produced by the gastric mucosa. Moreover, PAP-A3 and its fragments IMY25 and FLK22 possess intriguing anti-biofilm activities. In static growth conditions, recombinant peptide IMY25 can kill cells in a preformed *Staphylococcus aureus* biofilm and induce the disaggregation of *Pseudomonas aeruginosa* biofilm. Recombinant PAP-A3 and FLK22 are also able to induce disaggregation of *P. aeruginosa* biofilms in continuous flow conditions. Collectively, a new class of unexplored antimicrobial peptides, that may have the function to provide additional microbial surveillance within the human stomach and a potential to form the basis for novel effective anti-biofilm agents.

Among the fourteen synthetized lignan-like furan compounds, compound 11, namely methyl 4-(2-hydroxybenzyl)-2-phenylfuran-3-carboxylate, was the only one showing activity against *S. aureus* and *S. epidermidis*. The same compound resulted to be active against two clinical strains: Methicillin- resistant *S. aureus* (MRSA) and *S. epidermidis* (MRSE). Compound 11 also strongly reduced the biofilms vitality of *S. aureus* and *S. epidermidis*. This is a noteworthy feature since many antimicrobials are capable of disrupt the biofilm but not to eliminate biofilm bacteria, causing the release and the spread of live planktonic bacteria form the biofilm site. In conclusion, the antimicrobial activity of Compound 11 is of great interest, since it

can reduce both viability of planktonic as well as biofilm embedded bacteria.

Abietic acid has a good antibacterial activity against *S. pseudintermedius* strains suggesting it as a probable alternative antimicrobial compound. That said, the more relevant result here is its synergistic activity with Methicillin against MRSP. Abietic acid can therefore be beneficial in therapeutic applications for the elimination of micro-colonies, which are likely to exhibit increased resistance to Methicillin alone. Another advantage of Abietic acid is its anti-biofilm activity against both biofilm formation processes as well as against mature biofilms. These results highlight the potentiality of using Abietic acid as a starting point for the development of new antimicrobial agents. Future efforts should be focused on the study of the antimicrobial and anti-biofilm mechanism of action of this compound.

In conclusion, the new sources and technologies explored in this work for the creation of new antimicrobial compounds have all shown their potential to be decisive allies in the battle against the emerging antimicrobial resistance to conventional drugs. Already in this work it was shown that they have little or no hemolytic or cytotoxic activity at effective concentrations and, if these results will be confirmed by more detailed and thorough studies, they could also be introduced in the medical field for development of novel pharmaceutical agents.

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9. LIST OF PUBLICATIONS

List of publications

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8) Balato G., Roscetto E., Vollaro A., Galasso, O., Gasparini, G., Catania, M.R., Mariconda M. *Bacterial biofilm formation in antibiotic-loaded bone cement. A preliminary in vitro study.* (In preparation)