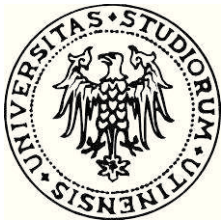

UNIVERSITÁ DEGLI STUDI DI UDINE

CORSO DI DOTTORATO IN SCIENZE BIOMEDICHE E BIOTECNOLOGICHE XXVI CICLO

TESI DI DOTTORATO DI RICERCA

EVALUATION OF THE THERAPEUTIC POTENTIAL OF NATURAL ANTIMICROBIAL PEPTIDES FOR HUMAN AND VETERINARY APPLICATION

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ANNO ACCADEMICO
2013-2014

ABSTRACT

Innovative antimicrobial drugs effective against emerging and often antibiotic-resistant pathogens are among the most challenging unmet medical needs. Natural antimicrobial peptides (AMPs) hold therapeutic potential as promising novel agents against bacterial and fungal pathogens [1] due to their biological activities including direct killing of invading microbes, modulation of the immune response and low frequency in selecting resistant strains [2]. The aim of the present thesis was to evaluate the functional and mechanistic properties of selected mammalian and fish AMPs in three different settings in view of their possible development as novel anti-infective agents for human and veterinary application.

1) In the first study three structurally diverse bovine peptides have been compared for their capacity to inactivate the yeast-like algae of the genus *Prototheca*. These microorganisms can cause mastitis in dairy cattle with big economic impact and no cure. The α-helical BMAP-28, the proline-rich Bac5 and the cysteine-rich lingual antimicrobial peptide (LAP) have been tested against a *P. wickerhamii* reference strain and against 12 clinical mastitis isolates in minimum inhibitory concentration (MIC) and time-kill assays. All AMPs were effective in the micromolar range. BMAP-28 sterilized *Prototheca* cultures within 30-60 min at its MIC, induced cell permeabilization with near 100% release of cellular ATP and resulted in extensive surface blebbing and release of intracellular material as observed by scanning electron microscopy. Bac5 and LAP inactivated *Prototheca* following 3-6 h incubation at four-fold their MIC and did not result in detectable surface damage despite 70-90% killing, suggesting they act via non-lytic mechanisms. Our results indicate that these three AMPs kill *Prototheca* with distinct potencies, killing kinetics and modes of action and may be appropriate for protothecal mastitis treatment. In addition, the ability of Bac5 and LAP to act via non-lytic mechanisms may be exploited for the development of target-selective drugs.

2) In the second study two alpha-helical AMPs, i.e. BMAP-28 and the human cathelicidin LL-37, were tested against *Candida* isolates from female genital tract infections. Vulvovaginal candidiasis affects approximately 75% of fertile age women with 5-10% incidence of recurrent infection. Importantly, the ability of *Candida* spp to form stable biofilms often results in serious medical device-related infections. The antifungal activity of these peptides was investigated against a reference *C. albicans*

strain and 24 clinical vaginal isolates of different *Candida* spp. Under standard experimental conditions, BMAP-28 was highly effective against all *Candida* isolates (MIC_{50}, 4 μM). Its antifungal effects were also preserved in synthetic vaginal simulated fluid (VSF) at vaginal pH values. The activity of these compounds was further tested against sessile *Candida* spp. by fluorescence microscopy and XTT reduction assays to quantify the inhibitory effect on biofilm cell viability. BMAP-28 strongly reduced the growth of mature biofilm through a lytic mechanism, whereas LL-37 could only prevent biofilm formation by inhibiting cell adhesion. Interestingly, both BMAP-28 and LL-37 inhibited *Candida* adhesion to medical grade silicone, suggesting a possible use of these peptides as antimicrobial coatings.

3) The immunomodulatory properties of AMPs have been addressed in the third part of my thesis, in view of developing salmonid AMPs as immunostimulants or vaccine adjuncts to prevent aquaculture infections. For this purpose, I analyzed the effects of BMAP-28 and of the salmonid cathelicidin STF on the respiratory burst and the pro-inflammatory cytokine genes expression in primary head kidney leukocytes from rainbow trout, by using a luminometric method and RT-qPCR analysis, respectively. The oxygen species production (ROS) in Phorbol-myristate-acetate- (PMA) and β-glucan- stimulated cells was synergistically increased in the presence of micromolar concentrations of each peptide. Moreover, the trout derived STF was able to speed up the respiratory burst kinetics in primary head kidney leukocytes, either stimulated with PMA or with β-glucan, and was also effective when added alone, though to a lesser extent. Real time PCR studies revealed a synergistic effect on IL-1β expression following four hour incubation of cells with STF and lipopolysaccharide, whereas no synergy was observed when the cells were incubated with the combination of each peptide with β-glucan. Each peptide alone did not affect cytokine genes expression and was not toxic to the cells, as assessed by measuring the release of lactate dehydrogenase in the medium.

Collectively, the results obtained highlight the antimicrobial and immunomodulatory properties of the peptides under study pointing out the differences in potency, kinetics, and mode of action of each single peptide, thus encouraging further studies in view of the development of these molecules for human and veterinary application.

INDEX

Abbreviations

AMB	amphotericin B
AMP	antimicrobial peptide
CD	circular dichroism
CFU	colony forming unit
ESI-MS	electrospray ionization-mass spectrometry
LAP	lingual antimicrobial peptide
LUV	large unilamellar vesicle
MIC	minimum inhibitory concentration
MFC	minimum fungicidal concentration
PC/SM/Er	L-α-phosphatidylcholine/sphingomyelin/ergosterol
PG/dPG	phosphatidyl/diphosphatidyl-DL-glycerol
SEM	scanning electron microscopy
TFE	trifluoroethanol
FLC	fluconazole
ITC,	itraconazole
MCZ	miconazole
PBS	phosphate buffered saline
VVC	vulvovaginal candidosis
HKL	head kidney leukocytes
ROS	Reactive oxygen species
RTG-2	fibroblast-like cell culture derived from gonad of normal rainbow trout (Oncorhynchus mykiss)
LPS	lipopolysaccharide
PMA	phorbol-12-myristate-13-acetate
HBSS	Hank's balanced salt soluton
LDH	Lactate dehydrogense

1 GENERAL INTRODUCTION

1.1 An overview of the innate immune system

Immunity refers to the global ability of the host to resist the predation of microbes that would otherwise destroy it. The fundamental feature of the immune system is the ability to distinguish between self and non-self. This is necessary to protect the organism from invading pathogens and to eliminate modified or altered cells. Although immunity has multiple facets, the greatest dichotomy separates two immune systems, innate and adaptive. The innate immune system is an ancient system found in all eukaryotes that acts as the first line of defense against invading organisms [1]. To the other hand, adaptive immune system acts as a second line of defense and also affords protection against re-exposure to the same pathogen. This type of immunity is typical to higher vertebrates and requires from a few days to a few weeks to be activated. Although in the past innate and adaptive immunity have been studied as separate defence mechanisms, in these years they are appreciated as obligate and synergistic parts of the system that mediates successful host responses to infection and tissue injury. Innate immunity not only provides the early defence capable of controlling and even eradicating pathogens before adaptive immunity becomes active, but is also required to alert, initiate and shape adaptive responses. It is accepted that the dependency of adaptive immunity on innate immune cells arise from the need for antigen presentation, a function displayed by antigen-presenting cells, including dendritic cells that form the main interface between the two systems. On the other hand, adaptive immunity often uses mechanisms of innate immunity to eradicate infections, reflecting a constant and bidirectional cross-talk between the two immune systems.

The innate immune system consists principally of three defensive elements: chemico-physical barriers, cellular elements and humoral elements. Chemico-physical barriers include the physical barrier function of the epidermis and mucosa, along with physiological functions such as cilial action, motility, desquamation and mucus secretion [2]. Cellular elements include epithelial cells, mast cells, dendritic cells, phagocytes such as macrophages and granulocytes, and lymphocytes such as NK cells and T cells [3]. The humoral elements of innate immunity are generically

subdivided into three subcomponents: soluble pathogen sensing molecules (e.g. natural antibodies; collectins such as surfactant proteins A and D and mannose-binding lectin; pentraxins; the lipopolysaccharide-binding protein (LBP)), cytokines and chemokines that orchestrate the immune response, and effector molecules that either directly kill microbes or activate defensive responses, such as the complement system, bactericidal proteins (e.g. lactoferrin, lysozyme, bactericidal permeability increasing factor – BPI, cathepsin G, CAP37/azurocidin) and antimicrobial peptides (e.g. defensins, cathelicidins, dermcidin) [2].

The important ability of the innate immune system to recognize and limit microbes early during infection is based primarily on the activation of pattern recognition receptors (PRRs), which detect distinct evolutionarily conserved structures on pathogens. These elements have been termed pathogen-associated molecular patterns (PAMPs) and include structurally diverse molecules such as lipopolysaccharide (LPS), lipoproteins, peptidoglycan, lipoarabinomannan and oligosaccharides. The PRRs are found on many cells of the innate immune system including epithelial cells, macrophage-monocytes, granulocytes, mast cells and dendritic cells. As opposed to the antigen receptors of adaptive immunity, which are encoded exclusively by rearranging members of the Ig superfamily, innate immune recognition is mediated by members of several protein families such as scavenger receptors, Toll-like receptors (TLR), formyl peptide receptors, mannose and glycan receptors, complement receptors (CR3) and CD14. There are also several soluble receptors, which bind PAMPs and some of these, such as CD14, are also found associated with cells [1,2,4]. Although PRRs were initially defined as cellular receptors, this definition has been then expanded to include secreted and locally produced molecules (e.g. the aforementioned collectins and pentraxins), that mediate different steps in inflammatory and immune responses including activation of complement or coagulation cascades, pathogen opsonization for phagocytic clearance and induction of inflammatory signaling pathways .

The family of TLRs is the major and most extensively studied class of PRRs and have been found in plants, insects and mammals [5]. Structurally, TLRs are integral membrane glycoproteins characterized by an extracellular or luminal ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain. Ligand binding to TLRs through PAMP-TLR interaction induces receptor oligomerization, which subsequently triggers intracellular signal transduction. To date, 10 TLRs have been identified in

humans with differing ligand-binding specificities [4]. For example, gram-negative bacteria are recognized by TLR4 via the lipid A portion of LPS, whereas lipoteichoic acid, lipoproteins, and peptidoglycan of gram-positive bacteria are detected by TLR2 [6] However, most gram-positive and -negative bacteria can activate additional TLRs via alternative PAMPs present in the cell membrane, cell wall, or intracellularly. To the other hand, TLRs are not able to recognize intracellular cytosolic pathogens and their derivatives, such as viral ssRNA, dsRNA, and DNA, as well as components of internalized or intracellular bacteria. It was shown that TLR-independent recognition of pathogens is mediated by a large group of cytosolic PRRs, which can be broadly divided into interferon (IFN)-inducible proteins, caspase-recruiting domain (CARD) helicases and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [4]. The former two families recognize viral nucleic acids and trigger antiviral immunity by inducing the production of type I interferons, while NLRs are activated by bacterial components such as muramyl-dipeptide and meso-diaminopimelic acid and mediate antibacterial immunity by activating NF-κB or through the formation of inflammasomes. The latter complexes lead to the activation of pro-inflammatory caspases, which control the processing and activation of the pro-inflammatory cytokines IL-1β and IL-18 [7].

The immune system uses many mechanisms to combat infection by microbes and to avoid coincidental damage to self-tissues. Among these, AMPs have recently found increased interest due to their pleiotropic function to not only kill pathogens but also control host physiological functions such as inflammation, angiogenesis and wound healing [8]. Consequently, AMPs are receiving great attention as promising alternatives to conventional antibiotics to overcome the current drug resistance crisis [9].

1.2 Antimicrobial peptides

1.2.1 General features

An important component of the humoral innate immunity is represented by the antimicrobial peptides (AMPs), molecules able to inhibit the microbial growth. AMPs are widely distributed in the plant and animal kingdom [10] and to date have well been identified over 2000 different sequences [11] derived from insects [12], molluscs [13], crustaceans [14], amphibians [15], fish [16], birds [17] and mammals [18]. In plants,

antimicrobial peptides have been found in seeds, leaves and other structures whereas in invertebrates they have been found in the haemolymph and in cytoplasmic granules of haemocytes. In vertebrates, they are present in all anatomic regions most prone to exposure to the external environment, such as skin, eyes, ears, and mucous membranes of the respiratory, gastro- intestinal and urogenital tract [19]. They are produced both by epithelial cells and by specialized cells of the innate immune system, including phagocytes. Phagocytes store AMPs in granules, mainly as inactive precursors, and release them within phagosomes or in the extracellular space upon cell activation. Some peptides are constitutively expressed, while others are upregulated upon infection or inflammation [20]. The majority of AMPs have a substantial proportion (≥ 30%) of hydrophobic residues and a net positive charge (generally +2 to +9) that promotes the interaction with prokaryotic membranes. Indeed, most AMPs exert their activity by directly acting on the membrane of pathogens as a consequence of their amphipathic nature. The first step in this activity is the initial attraction between the peptide and the target cell, which is thought to occur through electrostatic bonding between the cationic peptide and negatively charged components present on the outer bacterial envelope, such as phosphate groups within the lipopolysaccharides of Gram-negative bacteria or lipoteichoic acids present on the surfaces of Gram-positive bacteria. Then, the hydrophobic portions of AMPs are responsible for the interaction with hydrophobic components of the membrane. This complex interaction lead to rearrangements of membrane structure, which may result from the formation of peptide-lipid specific interactions, the peptide translocation across the membrane and interaction with intracellular targets or the most common mechanism, a membranolytic effect [21].

Noteworthy, the role of endogenous AMPs is not limited to the only direct inactivation of the micro-organisms, but is also extended to other important functions including modulation of the immune response and wound healing [22]. A peculiarity of antimicrobial peptides, that ensures a reduced toxicity to the host cells, is the ability to discriminate between prokaryotic and eukaryotic cells according to the existing differences at membrane level. In Gram-negative bacteria, both the outer layer of the plasma membrane as well as the outer membrane exhibit anionic molecules, while in the cell membranes of mammals the anionic lipid molecules are oriented towards the cytoplasmic side. Furthermore, contrary to bacteria, the membranes of eukaryotic cells are rich in cholesterol which is less prone to bind the AMPs [23]. Low toxicity toward

the “self” is also ensured by the compartmentalization of antimicrobial peptides in specific cell structures and a precise activation process.

In addition to typical AMPs, other endogenous molecules have been observed to exert antimicrobial activity, including certain cytokines and chemokines (e.g. CXCL4/PF-4, CCL5/RANTES, CCL-20/MIP3α, IFN-γ-inducible chemokines), selected neuropeptides and peptide hormons (e.g. neuropeptide Y, substance P, α-MSH, adrenomedullin), proteins such as lysozyme, RNAse-7, psoriasin and histons, as well as fragments of larger proteins (e.g. lactoferricin from lactoferrin, casocidin I from casein and fragments from bovine α-lactalbumin, human hemoglobin and ovalbumin) [24]. Damage-associated molecular patterns (DAMPs, danger signals, or alarmins) are also endogenous molecules endowed with antimicrobial activity. They are rapidly released by leukocytes and epithelial cells following uncontrolled cell death (passive release) or infection (secretion) and are able to recruit and activate antigen-presenting cells to enhance innate and adaptive immune responses [25].

1.2.2 Classification

The diversity of antimicrobial peptides discovered is so great that it is difficult to categorize them except broadly on the basis of their secondary structure. Moreover, the taxonomical classification of antimicrobial peptides has not been considered properly adequate as a consequence of the structural patterns similarity shared by peptides from widely different organisms. To the other hand, a classification based on chemical-structural criteria is considered quite useful for cataloguing the different types of AMPs [26]. The classification defines two broad groups: linear peptides and cyclic peptides (Fig 1.1). The first group of peptides are divided according to the tendency to adopt particular three-dimensional structures, such as amphipathic α-helices, or to the predominance of particular residues (Arg, Pro and occasionally Trp). The second group includes all cysteines-containing peptides and can also be divided into more subgroups corresponding to single or multiple disulfide structures.

All the known antimicrobial peptides are made from gene-encoded precursors (prepropeptides), from which the mature peptides are derived by the sequential removal of the signal peptide and of a variably extended prosequence. In general the propiece precedes the mature peptide, is anionic and, at least in some cases, has been suggested to play a role in targeting and/or in assisting the correct folding of the antimicrobial peptide [27]. The preproregion is often highly conserved within families of antimicrobial peptides, as deduced from sequence analysis of the precursors at the

cDNA level, thus suggesting that members of each family evolved from ancestor genes through duplication and modification [28]. Among the discovered families of AMPs, cathelicidins and defensins have been very well studied families as a consequence of their wide distribution in living organisms.

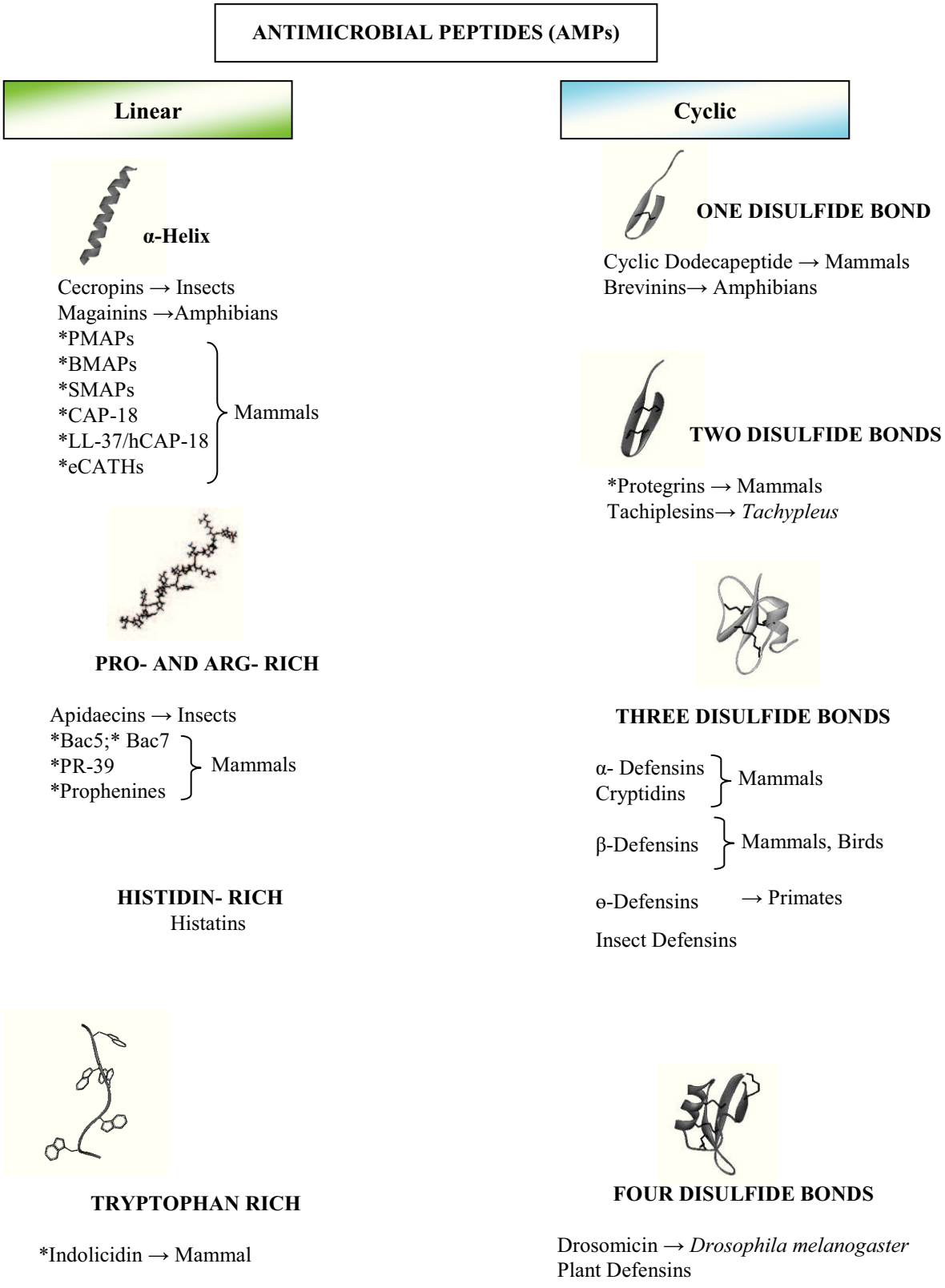

Figure 1.1 Examples of AMPs classified according to their structural characteristics. *denotes cathelicidin derived peptides.

The mammalian defensins are cationic peptides, generally not glycosylated, relatively rich in arginine, with a molecular mass from 3.5 to 4.5 kDa. They are synthesized as 93–96 amino acid prepropeptides containing a 19-residue signal sequence and a 40- to 45-residue anionic proregion. The prodomain is followed by the antimicrobial peptide, which is generated from the precursor molecule by proteolytic processing. One of the main features of these families of AMPs is the presence of six highly conserved cysteine residues, forming three intramolecular disulfide bridges. However, The role of the disulphide bridges in conferring antimicrobial activity to these AMPs is still debated. For example, the disulphide-bridged-β-sheet present in the porcine protegrins was seen to be a key requirement to the antibacterial activity and stability of these peptides [29] whereas Schroeder BO. et al have recently observed that the activity of the human β-defensin-1 was somewhat preserved in the presence of reduced disulfide brides [30]. Based on the organization of these intramolecular disulphide bonds between cysteine residues, the vertebrate defensins are divided into two major classes: α-defensins and β-defensins are present in mammals, birds and reptiles [31]. In some primates, but not in humans, are also present the θ-defensins, with a cyclic peptide chain, derived from α-defensins [32]. In humans, the main cellular source of α-defensins are neutrophils (human neutrophil defensins (HNP)- 1-4) and intestinal Paneth cells (human defensin (HD) 5-6), while β-defensins are primarily produced by epithelial cells, monocytes/macrophages and dendritic cells [33]. When used in *in vitro* assays at micromolar concentrations, defensins kill a wide variety of gram-positive and gram-negative bacteria and fungi [34-36]. In addition, some defensins are effective against enveloped viruses, such as herpes simplex [35]. In general, defensins require low salt concentrations for their activity, which is enhanced in the absence of Ca^{2+} or Mg^{2+} [35]. Studies of antimicrobial mechanisms of defensins have shown that they permeabilize the outer and inner membranes of Escherichia coli. After initial electrostatic binding to negatively charged molecules (head groups of polar membrane lipids) on the target cell surface, they insert into the energized cell membrane and seem to form multimeric ion-permeable channels in a voltage-dependent manner [35]. In addition to the direct antimicrobial activity, defensins appear to have also diverse immune-related functions. It was seen that Human beta defensin-2 promotes histamine release and prostaglandin D2 production in mast. Moreover, it was seen that defensins are chemotactic for monocytes, polymorphonuclear leucocytes and T-Cells [37,38].

Cathelicidins are often expressed by cells in direct contact with the environment such as epithelial cells of the intestine, airway, genitals, ocular surface, skin and in eccrine glands in which they form the first layer of defence against pathogens [39]. To date, about 30 cathelicidin family members have been identified in animal species including rabbits [40], pigs [41], cattle [42], sheep [43], humans [44], guinea pigs [45], mice [46], goats [47], horses [48], monkeys [49], rats [50], rainbow trout and atlantic salmon [51,52], dogs [53], donkeys [54], cats [55] and Pandas [56].

The peptide family named "cathelicidins" with a common proregion (cathelin domain) was first identified in mammals in bone marrow myeloid cells [57]. The term derives from the high homology of the conserved N-terminal region with cathelin, a protein that was isolated from porcine neutrophils as inhibitor of cathepsin L before the initial identification of the cathelicidin family [58]. The members of these family are synthesized as modular proteins of 16-26 kDa, in which the C-terminal antimicrobial domain is connected to a N-terminal pre-proregion including a putative signal peptide of approximately 30 amino acids and a highly conserved propiece (Fig. 1.2) [57,59]. The signal peptide and the cathelin domain are coded for in exon I through exon III while the exon IV region contains the processing site, the antimicrobial peptide-coding sequence and the 3'UTR. The sequence corresponding to the mature antimicrobial peptide within the exon IV region is the only hypervariable sequence in the gene and seems to represent a precise target for evolutionary diversification varying in amino acid sequence, structure and size [28]. Indeed, the peptides derived from exon IV are stored in the secretory granules of neutrophils and can be released extracellularly upon leucocyte activation. To date, the cathelicidin derived peptides can be classified in five distinct groups according to their structural characteristics:

- Cyclic peptides with one disulfide bond, such as the cyclic dodecapeptide in cattle.
- Porcine protegrins with two disulfide bonds.
- Peptides containing a high number of tryptophan residues such as indolicidin
- Peptides with α-helical structure such as bovine BMAP-27, -28 and -34, ovine SMAP-29 and -34, porcine PMAP-36 and -37, human LL-37/hCAP-18, rabbit CAP-18 and mice CRAMP.
- Short sequences arranged in tandem repeats such as bactenecins (bovine Bac5 and Bac7, ovine OaBac5 and 7.5 as well as porcine PR-39 and prophenins)

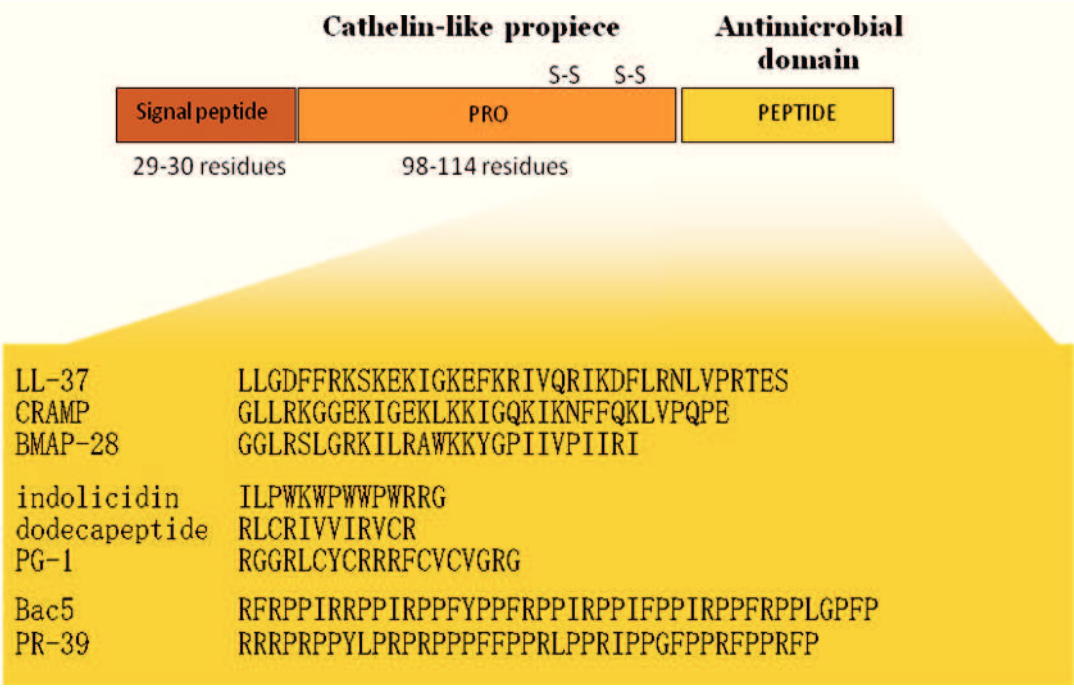

Figure 1.2 The Structural diversity of the antimicrobial peptides derived from the C-terminal domain of the mammalian cathelicidin precursors.

Mature cathelicidin peptides generally exhibit broad-spectrum antimicrobial activity against a range of Gram-positive and Gram-negative bacterial species. It has been shown that several cathelicidins are also effective at killing nosocomial isolates of *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* that are resistant to conventional antibiotic therapy [60]. Moreover, these peptides were also active against certain fungi (e.g. *Candida albicans* and *Cryptococcus neoformans*) [61], against parasites (e.g. *Cryptosporidium parvum* and *African trypanosomes*) [62,63], or enveloped viruses (e.g. human immunodeficiency virus) [64,65]. With few possible exceptions (proline-rich peptides and the loop form of dodecapeptide), the killing mechanism is mediated by disruption of the integrity of bacterial membranes.

Despite the large amount of beta defensin (hBD) genes in human genome [66], there is only one cathelicidin gene (CAMP) identified in humans. CAMP encodes the peptide LL-37 which begins with two leucine residues at its N-terminus, and is 37 a.a residues long. hCAP18/LL-37 is expressed in various cells and tissues such as circulating neutrophils, myeloid bone marrow cells, epithelial cells of the skin and is also expressed in the gastrointestinal tract, as well as in the epididymis and lungs [67]. Structurally, LL-37 forms an amphipatic, cationic α-helix in aqueous solution that permits the incorporation into lipid bilayers, which leads to disruption of microbial membranes, viral envelopes and some fungal structures [68].

Regarding the activity of this peptides, it has been shown that LL-37 is able to inhibit the growth of a variety of Gram-negative (*P. aeruginosa*, *S. typhimurium*, *E. coli*) and Gram-positive (*S. aureus*, S. epidermidis, *L. monocytogenes* and vancomycin-resistant enterococci) species. Moreover, LL-37 is also active against clinically important strains of Gram-negative uropathogens (*E. coli* HU734, *P. aeruginosa* AK1, *K. Pneumoniae* 3a), periodontal (*Actinobacillus actinomycetemcomotans*, *Capnocytophaga*, *Porphyromonas* and *Prevotella* spp.) and common wound pathogens (Group A Streptococcus) [59]. The high amounts of LL-37 detected in the airway surface fluid during infection and especially in psoriatic skin lesions [69,70], suggest that the concentrations of LL-37 that are effective *in vitro* are fully compatible with those found *in vivo*. Further, unlike most defensin members, LL-37 is active against several bacteria in high-salt media (up to 150 mM), supporting its capacity to function under a variety of physiological conditions . This peptide also exhibited antifungal activity against *Candida albicans*, but in more strict conditions and with strong dependence on the culture medium. Further, LL-37 has been shown to inhibit the replication of HIV and vaccinia *virus in vitro* [71]. Next to its broad antimicrobial function, LL-37 is able to signal tissue and cell damage. This results in the chemoattraction, as well as stimulation and modulation of cytokine release from cells of the innate and adaptative immune system. Thus, LL-37 can be viewed as an alarmin molecule [39].

In contrast with a single member of cathelicidins present in humans, a variety of these peptides are found in cattle. The first bovine cathelicidin isolated from neutrophils were bactenecins 5 and 7, whose names come from the term bactenecin and an indication of the apparent mass, respectively 5 and 7 kDa [72]. At difference to other types of AMPs, these peptides have the ability to penetrate into bacterial cells without causing membrane permeabilization and, once in the cytoplasm, inhibit the activity of specific molecular targets essential for bacterial growth, leading to cell death.

Along with bactenecins, the bovine peptides BMAP-27 and BMAP-28 were also been isolated from bovine neutrophils. It has been shown that BMAPs rapidly permeabilize and kill *in vitro* a wide range of bacteria and fungi in the low micromolar and submicromolar range of peptide concentrations [73]. BMAPs are haemolytic *in vitro* at concentrations that are approximately tenfold higher than those microbicidal and they are toxic for mammalian tumor cells, inducing their apoptosis. Moreover, BMAPs have also been found cytotoxic against other mammalian cells [74]. Apart from

direct bacterial killing, BMAPs posses also other biological activities that also include modulation of immune responses to infections [75,76]. Regarding BMAP-28 , it has been shown that peritoneal injection of this peptide in mice promotes both neutrophil recruitment and increased phagocytic ability [77].

1.3 Need for novel antimicrobials

Antimicrobial drugs have been widely used in human and veterinary medicine for more than 50 years, with tremendous benefits to both human and animal health. The development of resistance to this important class of drugs, and the resulting loss of their effectiveness as antimicrobial therapies, poses a serious public health threat.

These problems are of particular relevance in healthcare-associated infections including those acquired in intensive care units, surgical sites and those related to the use of medical devices. In 2009, the European Centre for Disease Prevention and Control (ECDC), the European Medicines Agency (EMEA) and the international network Action on Antibiotic Resistance (ReAct) have documented the gap between the frequency of multidrug-resistant bacterial infections in Europe and the development of new antibiotics. For this study, antibiotic-resistant bacteria such as methicillin resistance *Staphylococcus aureus* (MRSA), third-generation cephalosporin resistant *Enterobacteriaceae* (e.g. *Escherichia coli*, *Klebsiella pneumoniae*), and carbapenem resistant Enterobacteriaceae (e.g. *K. pneumoniae*), were selected because they are frequently responsible for bloodstream infections and because the associated antibiotic resistant trait is, in most cases, a marker for multiple resistance. In this regard, the average proportion of MRSA was the highest proportion of antibiotic-resistant isolates among the selected bacteria frequently responsible for bloodstream infections in the European Union. Moreover, it has also been shown that the average proportion of *Escherichia coli* blood isolates showing resistance to third-generation cephalosporins has been rising steadily, whereas the proportion of *K. pneumoniae* blood isolates from EU Member States that showed resistance to carbapenems was, in general, very low [78]. Antimicrobial resistance is not limited to bacteria but it also applies to fungal pathogens, with high morbidity and mortality rates especially in immunocompromised patients. Antimicrobial resistance represents a current issue also in cystic fibrosis patients where, although antibiotics are still an effective tool for treating bacterial lung infections, the alarming rise of multidrug-

resistant bacteria has highlighted the need for new therapies. Indeed, it has been shown that the therapy of cystic fibrosis pulmonary infection is hampered by several factors including broad-spectrum antimicrobial resistance exhibited by bacterial pathogens, poor penetration of antibiotics towards the site of infection and the ability of microorganisms to form sessile microbial communities called biofilms that are intrinsically resistant to antibiotics and host immune responses. Taken together, these factors lead to a progressive and irreversible airway damage from which most patients ultimately die [79].

However, in spite of the rise of resistant pathogens, the rate of new antibiotics is dropping. Since the early 1960s, only four new classes of antibiotics have been introduced (Figure 1.3), and none of these have made a major impact yet, whereas the ~$30 billion global antibiotics market is still dominated by antibiotic classes discovered half a century ago. Indeed, all antibiotics approved for clinical use between the early 1960s and 2000 were synthetic derivates of existing core structures [80].

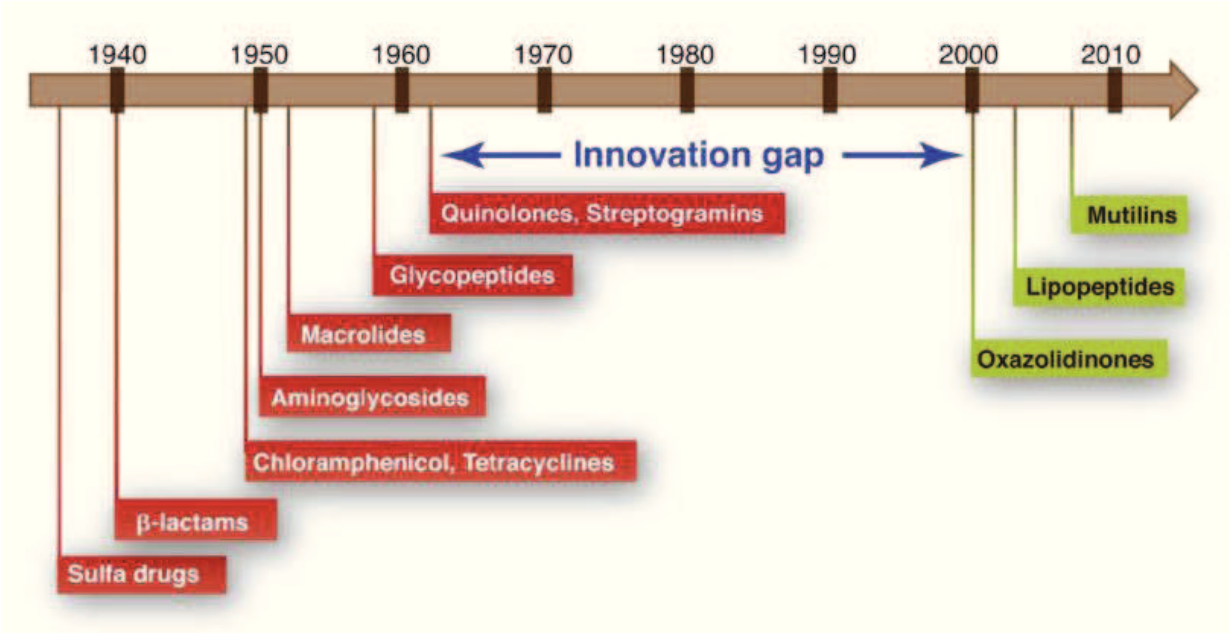

Figure 1.3 Between 1962 and 2000, no major classes of antibiotics were introduced (Fischbach MA and Wals CT. Science. 2009)

Moreover, two factors exacerbate the problem of drug resistance by creating unique dis-incentives for antibiotic development. First, antibiotics are used in smaller quantities than other drugs. Prescriptions for chronic illnesses can last years or decades, whereas a standard course of antibiotics lasts only weeks; therefore, antibiotics yield lower revenues than most drugs. Second, whereas most newly approved drugs can be prescribed to all who would benefit, the use of a newly approved antibiotic may be restricted to the treatment of serious bacterial infections.

The result is that the resistance is on the rise while antibiotic discovery and development are on the declin [80]. In order to find suitable solutions against antimicrobial resistance, the European Commission has recently adopted a 5-year action plan [81] that includes a new research programme supporting antimicrobial development.

Thus, the development and approval of new compounds for clinical applications is of great importance. Among these compounds, AMPs appear to be excellent candidates for the development of novel antimicrobial agents. These molecules are endowed with several desirable properties, including potent and broad-spectrum antimicrobial activity also towards multi-drug resistant microbial isolates, and scarce propensity to raise resistant mutants due to fast membranolytic mechanism of action [82]. In addition to their antimicrobial action, AMPs have demonstrated diverse biological effects, all of which participate in the control of infectious and inflammatory disease, characteristics that make these peptides attractive as therapeutic tools. (Fig 1.4)

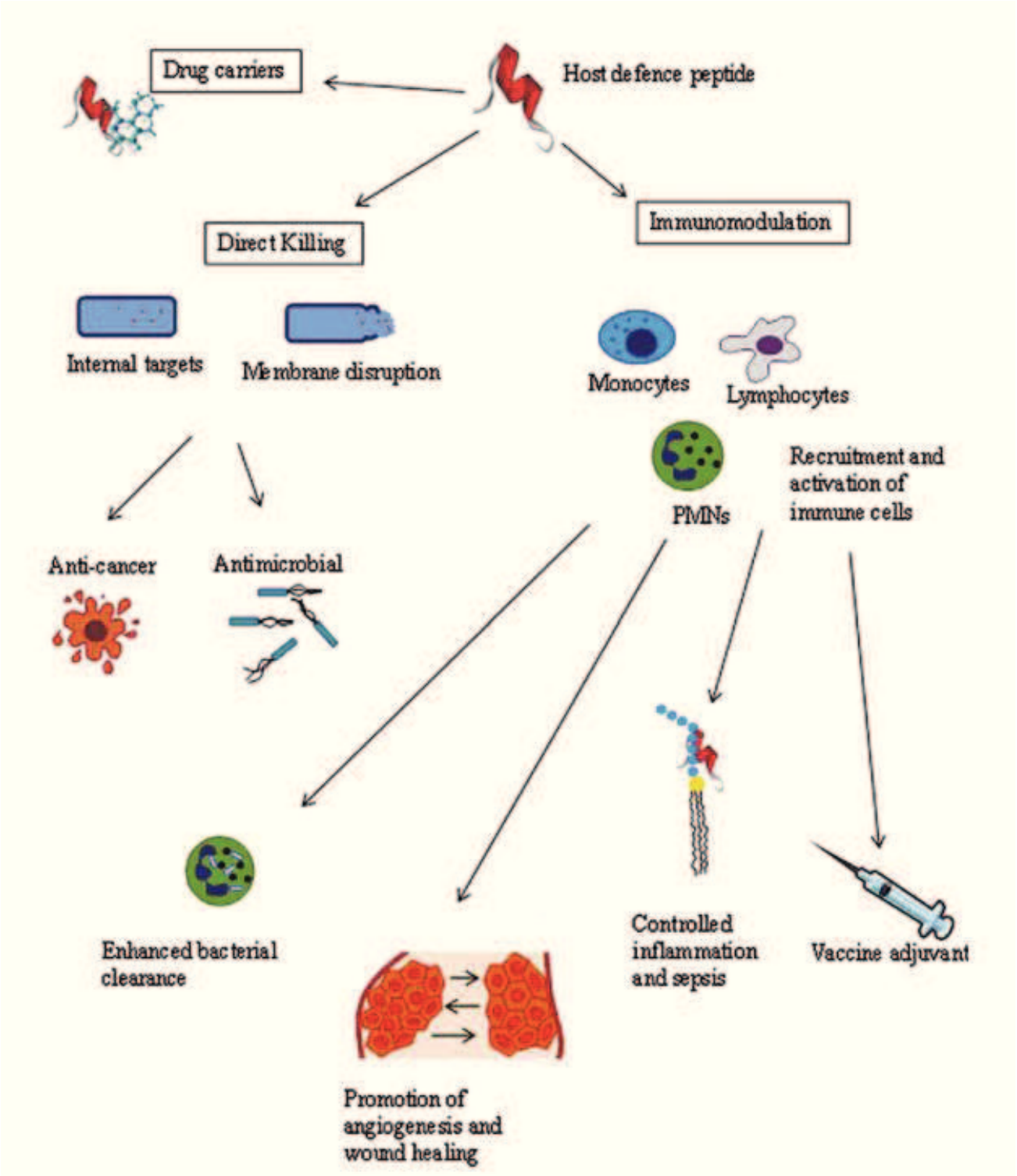

Figure 1.4 Biological properties of AMPs and potential applications. (Yeung Amy TY and Hancock REW. 2011)

1.4 An update on the therapeutic potential of AMPs

1.4.1 AMPs as antimicrobial and anti-inflammatory agents

There are several potential advantages to using AMPs as antimicrobial drugs over conventional antibiotics. They can be used as antibiotics alone, or in combination with other antimicrobials for a synergistic effect. AMPs demonstrate broad-spectrum activity against bacteria with a bactericidal mechanism rather than bacteriostatic one. Increasing evidence demonstrates that AMPs have multiple targets within the bacterial cell [24]. As a result of their amphipathic nature, all AMPs interact directly with the cytoplasmic membrane of Gram-positive and Gram-negative bacteria, as well as eukaryotic microbes [83] leading to either membrane barrier disruption or to peptide uptake and inhibition of intracellular targets. Some alternative targets include the macromolecular synthesis, cell division, cell wall biosynthesis and certain heat shock enzymes. This multiple targeting and interaction with fundamental physiological structures makes microbes less likely to develop resistance [84].

Despite the very substantial number of antimicrobial peptides that have been identified and their recognized potential as antibacterial, relatively few have actually proceeded into clinical trials based on promising data from *in vitro* and animal studies [85]. Examples of peptides at their most advanced stages of clinical development as antimicrobial and anti-iflmmatory agents are shown in Table 1.1. Pexiganan, topically administered to sufferers of diabetic foot ulcer, was as effective as oral antibiotic treatment with ofloxacin, leading to a clinical cure or improvement in 90% of patients. Before the nonapproval letter of FDA in 1999 for the use of pexiganan as topic treatment of diabetic foot ulcers, this peptide was developed by Magainin Pharmaceuticals, a biotech company that is now defunct, and SmithKline Beecham (which later became GlaxoSmithKline; GSK). Actually, the pharmaceutical company Locitex under the sponsorship of Dipexium Pharmaceuticals is negotiating with FDA to bring pexiganan through clinical trials, as a way of treating bacterial infections associated with diabetic ulcers, particularly when the bacteria are resistant to standard antibiotics. Omiganan is a synthetic cationic peptide, derived from indolicidin, developed by BioWest Therapeutics (Vancouver). Currently, phase 2 trials are under way for the development of this peptide for the treatment of inflammatory papules and pustules associated with rosacea.

Novexatin is a cyclic and highly cationic (arginine-rich) peptide based on human α and β defensins developed by the pharmaceutical company NovaBiotics of Aberdeen (UK). Currently, phase 2 trials are under way for the development of this peptide for the treatments of fungal infections in the toenails.

OP-145, the lead compound of the biopharmaceutical company OctoPlus (Leiden, The Netherlands), is a syntetic 24-mer peptide, derived from LL-37, for binding to lipopolysaccharides or lipoteichoic acid. Currently, phase 2 trials are under way for the development of this peptide for the treatments of chronic bacterial middle-ear infections. Oragenics is another low-profile company developing antimicrobial peptides as anti-inflammatory agents. In this case, its lead compound MU1140, is a peptide belonging to the class of lantibiotics: peptide antibiotics, both linear and looped, so called for the lanthionine (polycyclic thioether) amino acids they contain. In mid-2012, Oragenics teamed up with Intrexon corporation of Germantown to produce and develop this and another lantibiotic for human but also for veterinary medical uses [11].

1.4.2 AMPs as antimicrobial coatings

The use of biomaterial implants and medical devices such as catheters, heart valves, stents, shunts, arthroprostheses and fracture fixation devices is an increasingly common and often life-saving procedure. Although infection incidence has been reduced by aseptic surgical techniques and prophylactic systemic antibiotic therapy, pathogen colonization of medical devices or implants still represents a serious problem [86]. Indeed, the presence of microbial communities called biofilms (see chapter 3 of the thesis) form environments that enhance antimicrobial resistance leading to a reduction in the effectiveness of current therapies. As a result, there is a continuous search to overcome or control such problems, leading researchers to find new kinds of coating agents. Among them, AMPs have received increased attention owing to their broad spectrum antimicrobial activity also against microbial biofilms, good biocompatibility, and, above all, the low frequency in selecting resistant mutants [87]. To date, several AMPs have been tested in animal models and in preclinical trials as coating material to prevent colonization of indwelling medical devices with promising results.

Citropin 1.1 is a highly hydrophobic, 16 amino acid peptide produced by both the dorsal and submental gland of the green tree frog *Litoria citropa* that possesses a wide-spectrum antimicrobial activity [88]. Using a rat model of central venous catheters

infection, Cirioni O. et al. studied the efficacy of Citropin 1.1, alone or in combination with two hydrophobic antibiotics (minocycline and rinfampicin) in the treatment of central venous catheters associated S aureus infection [89]. The results obtained demonstrated that treatment of catheter with this peptide or with high dose of antibiotics, but not with a low dose, caused a significant reduction of the biofilm bacterial load and bacteremia as compared to the control group [90]. Similar results were obtained when the bovine antimicrobial peptide BMAP-28 and the protegrin derivate IB-367 were tested in the same rat model of central venous catheters-related infections [91].

Omiganan, currently under development for the treatment of rosacea, was originally developed to prevent catheter colonization and microbiologically confirmed infections, reaching phase 3b studies before the nonapproval letter from the FDA for this application. To date, it is not fully understood why Omiganan have not reached the approval of the FDA. J.L Fox. 2013 have suggested that this decision is probably due to the scarce propencity of FDA to approve new antibiotics in these years [11]. Omiganan has been demonstrated to exert a rapid and broad spectrum bactericidal activity against a large collection of gram-positive and gram-negative clinical isolates commonly responsable for catheter-associated infections, including strains with emerging resistant phenotypes like MRSA, vancomycin-resistant enterococci and extended-spectrum β-lactamase *E.coli* [92]. Importantly, the same peptide was also demonstrated to be active against contemporary fungal pathogens responsible for catheter associated infections [92] and to significantly reduce normal skin flora count following topical application.

The increased awareness of the possible advantages that may rise from the use of AMP-coated surfaces in the control of device-related infections has stimulated the researchers to identify the omptimal conditions for AMP adhesion to and/or release from synthetic surfaces [87]. The release strategy offers the potential for extended activity, but has to date failed to achieve delivery of a sustained and effective dosage over a relatively prolonged period of time. To address this issue, there has been an increased interest in covalent attachment of drugs to the implant surface. Covalent immobilization of the AMPs could increase their long-term stability while decreasing their toxicity, as compared to incorporation approaches on leach- or release-based systems [93]. To date, a wide variety of solid supports has been assessed for production of surfaces with immobilized AMPs, including polymeric brushes and resins [86], metal (e.g. silanized titanium) [94], glass coverslips [95], model surfaces (e.g.

self-assembled monolayers) [96] and even commercial contact lenses [97]. Using these solids supports, the effect of covalent immobilization of AMPs on their antimicrobial activity has been studied by several investigators [93]. For example, in 1995, Haynie et al. demonstrated that some AMPs (naturally occurring magainin 2 and several idealized synthetic amphipathic peptides) immobilized onto a polyamide resin (pepsin K) retained lethal activity against several Gram-positive and Gram-negative bacteria [98]. Willcox MD. et al. compared the antimicrobial activity of the synthetic peptide melimine that was adsorbed or covalently immobilized onto commercial contact lenses (Etafilcon A). An apparent increase in efficacy was observed when the peptide was covalently attached to the surface, which was ascribed to a possibly higher relative surface availability of the peptide, in contrast to the adsorption process where peptide aggregation could produce uneven peptide distribution [97].

1.4.3 AMPs as immunostimulants

To date, there is a considerable evidence about the capacity of several AMPs to modulate the immune response in addition to their direct killing effect. The immunomodulatory activities of AMPs are extremely diverse and include stimulation of chemotaxis directly and/or through chemokine production, suppression of bacterial induced pro-inflammatory cytokine production, regulation of neutrophil and epithelial cell apoptosis, modulation of cellular differentiation pathways, modulation of dendritic cell activation and differentiation, and promotion of angiogenesis and wound healing [84] .

The selective enhancement of innate immunity by cationic AMPs represents a novel approach to (adjunctive) anti-infective therapy that complements directly microbicidal compounds [99]. For example, the human cathelicidin LL-37 added exogenously to mice can protect against Gram-positive bacterial infections but is not able to reduce the bacterial load in tissue culture medium that contains physiologically relevant salt concentrations [100]. In contrast, at physiological concentrations of peptides, salt and serum, cationic AMPs such as LL-37, exhibit a wide range of alternative biological functions that do not target the pathogen directly, but rather selectively enhance and/or modulate host defence mechanisms to combat microbial infections [101]. Identifying how the peptides interact with and stimulate the cells of the immune system is one of the greatest obstacles in the development of immunomodulatory peptides as therapeutics. It has been shown that immunomodulatory peptides target multiple receptors and processes within cells,

depending on both the cell type and the amino acid sequence of the peptide. For instance, LL-37 is able to produce multiple effects through the activation of multiple receptors. This peptide is chemotactic for freshly isolated human peripheral blood neutrophils, monocytes, and T cells through the activation of the formyl peptide receptor-like 1 [102] and induces fibroblast proliferation through the activation of the P2X(7) receptor [103]. Apart from chemotactic effects and modulation of cytokine release, LL-37 also regulates apoptosis, promotes wound healing and neutralizes lipopolysaccharide (LPS) activities. To date, only three synthetic cationic peptides (variants of AMPs) have progressed to phase III clinical trials and despite evidence of efficacy for two of them, none have been approved for clinical use. Interestingly, several AMPs entered in clinical trials as antimicrobial, subsequently demonstrated immunomodulatory activity. An example is the peptide Omiganan that has demonstrated activity in phase III clinical trials in suppressing inflammation due to rosacea. Therefore, it is quite probably that the immunomodulatory activities play a substantial role in any clinical benefit these drug would demonstrate [84].

AMPs have been considered attractive candidates as vaccine adjuvants owing to their ability to modulate the immune system [84]. Evidence for adjuvant activities of AMPs is based on various observations. For example, human neutrophil defensins have been shown to enhance both humoral and cell-mediated antigen-specific immune responses in murine models whereas DNA vaccines encoding human immunodeficiency virus-1 glycoprotein 120, fused to murine β-defensin 2, induced systemic and mucosal immune responses [104]. A recent study showed that hBD-2 and hBD-3 exhibit strong adjuvant activities [105]. Similar to LL-37, hBD-2 and hBD-3 form aggregates with DNA. Together the hBD-2-DNA and hBD-3-DNA aggregates induce TLR-9-dependent interferon-α production in plasmacytoid dendritic cells [105]. To co-formulate AMPs into novel vaccines, several issues need to be considered, including the quality and type of immune response, compatibility with the antigen, safety, stability and cost.

Considering the biological activities of AMPs, it is not surprising that certain peptides exhibit also wound-healing properties *in vitro* and *in vivo*. The role of AMPs in wound healing is supported by the observation that AMPs, such as human cathelicidin (hCAP18/LL-37) and hBD-2 and -3, are highly expressed in epidermal keratinocytes in response to injury or infection of the skin [106]. For example, treatment with exogenous hBD-3 led to enhanced re-epithelialization of wounds in a porcine model [107]. In addition, AMPs have been demonstrated to stimulate the expression of

growth factors and cytokines in epithelial cells and keratinocytes that are also important in wound healing. For example, LL-37 induces the secretion of IL-18 from keratinocytes [108].

1.4.4 AMPs as drug carriers

The ability of several AMPs to penetrate into the cells without causing lysis of the membrane has prompted the researchers to consider these molecules as possible drug carriers seeing the difficulty of many drugs to cross the hydrophobic membrane owing to their generally hydrophilic nature [84]. Recently, the internalization of bioactive cargos into live cells has become an area of fast growth. In particular, cell-penetrating peptides (CPPs) have been identified as promoting agents in the transport of a wide variety of conjugated molecules across the membrane ranging from peptides to antibodies and drug-loaded nanoparticles. Noteworthy, the ability to introduce drugs into cells allows the alteration of the conventional drugs biodistribution, in order to reduce the toxicity and patient compliances. [109]. CPPs share the same fundamental characteristics of AMPs (short, cationic and often amphipathic) and it is supposed that they enter in the cell by several mechanisms including direct translocation across the plasma membrane and endocytosis [84]. The two most well known CPPs are TAT, derived from the trans-activating transcriptional activator (Tat) domain from human immunodeficiency virus 1 (HIV-1), and Penetratin, a 16-amino acid peptide derived from Drosophila transcriptional regulator antennapedia (Ant). TAT has been conjugated to several molecules with promising *in vivo* results whereas administration of the anticancer agent methotrexate to Pentatrin produce a fivefold increase in cytotoxicity in a breast cancer cell line [110].

Similarly, the antimicrobial peptide LL-37 is able to traverse into the cells, which is required for chemokine induction [111], and demonstrated a cytosolic receptor [112]. It can also carry passenger molecules into the cells. Indeed, Sandgren S. et al. 2004 have seen that LL-37·DNA complexes enter in mammalian cells via endocytosis [113].

1.5 Improvement of AMPs for Clinical Use

Despite considerable progress in the development of AMPs as therapeutics certain limitations remain, including cost of production, stability and toxicity *in vivo*. Ideally, peptide therapeutics should have a low cost of production but unfortunately the

current method of peptide production is quite expensive. Indeed, the cost price of synthetic peptides is five to twenty times as high as that of conventional antibiotics and typically runs in the range of US $ 100-600 per gram [114]. One of the cheapest alternatives to synthetic peptides is production by recombinant expression methods using microorganisms that are resistant to the produced peptide antibiotic. Plectasin, the first fungal defensin with an interesting therapeutic potential, can be produced in a fungal expression system effectively and at high yields. The same system is currently used for industrial scale production of other proteins by Novozyme [115]. Another way to address this issue would be to create truncated peptides with equivalent potencies, to be produced by synthetic methods.

A second and obvious disadvantage of AMPs is their potential suceptibility to proteases, which reduce their half-lives *in vivo*. In particular chymotrypsin-like enzymes attack proteins at basic residues, which are an obligate feature of antimicrobial peptides [114]. Peptide stability can be enhanced through the use of D-amino acids, alternative backbones (peptidomimetics) or synthetic amino acids, all of which are resistant to proteolytic degradation. However, all of these strategies unfortunately have a common drawback: increase the cost of production [22].

Finally, the toxicity of several AMPs on eukaryotic cells limit the use of these molecules as therapeutics. Indeed, the majority of clinical trials have involved topically instead of systemically applied peptides. Although AMPs bind to bacterial surfaces via electrostatic interactions, some types of AMPs can directly interact with host cells and lyse them [116]. To solve the issue of hemolysis, it is important optimizing the peptide sequences to decrease hemolytic activity. Many naturally occurring AMPs are amidated at the C terminus; amidated peptides exhibit higher antimicrobial activity but are also more hemolytic than that of natural AMPs [117]. Strandberg. E. *et al* reported that C-terminal deamidation of AMPs reduces undesired hemolytic activity while maintaining antimicrobial effects [118]. It is known that C-terminal amidation stabilizes amphipathic helix formation of AMPs upon their binding to the membrane bilayer, leading to strong activity.

Although certain limitations are apparent, the clinical potential of this group of molecules will undoubtedly be better understood by studying the functional and mechanistic properties of AMP in order to minimize the cost of production and improving the currently available formulation strategies.

Product	Description	Indication	Phase	Company (location)
Magainin peptide/ pexiganan acetate	22-amino-acid linear antimicrobial peptide, isolated from the skin of the African clawed frog (*Xenopus laevis*)	Diabetic foot ulcers	3	Dipexium Pharma (White Plains, New York)/MacroChem/Genaera
Omiganan	Synthetic cationic peptide derived from indolicidin	Rosacea	2	BioWest Therapeutics/Maruho (Vancouver)
OP-145	Synthetic 24-mer peptide derived from LL-37 for binding to lipopolysaccharides or lipoteichoic acid	Chronic bacterial middle-ear infection	2	OctoPlus (Leiden, The Netherlands)
Novexatin	Cyclic cationic peptide, 1,093 daltons	Fungal infections of the toenail	1/2	NovaBiotics (Aberdeen, UK)
Lytixar (LTX-109)	Synthetic, membrane-degrading peptide	Nasally colonized MRSA	1/2	Lytix Biopharma (Oslo)
NVB302	Class B lantibiotic	*C. difficile*	1	Novacta (Welwyn Garden City, UK)
MU1140	Lantibiotic	Gram-positive bacteria (MRSA, *C. difficile*)	Preclinical	Oragenics (Tampa, Florida)
Arenicin	21 amino acids; rich in arginine and hydrophobic amino acids	Multiresistant Gram-positive bacteria	Preclinical	Adenium Biotech Copenhagen
Avidocin and purocin	Modified R-type bacteriocins from *Pseudomonas aeruginosa*	Narrow spectrum antibiotic for human health and food safety	Preclinical	AvidBiotics (S. San Francisco, California)
IMX924	Synthetic 5-amino-acid peptide innate defense regulator	Gram-negative and Gram-positive bacteria (improves survival and reduces tissue damage)	Preclinical	Iminex (Coquitlam, British Columbia, Canada)

Table 1.1 Anti-microbial peptides in clinical trials or in development (Jeffrey L. Fox. 2013. Nature)

2 AIM OF THE STUDY

Innovative antimicrobial drugs effective against emerging and often antibiotic-resistant pathogens are among the most challenging unmet medical needs. Natural antimicrobial peptides (AMPs) hold therapeutic potential as promising novel agents against bacterial and fungal pathogens [119] due to their biological activities including direct killing of invading microbes, modulation of the immune response and low frequency in selecting resistant strains [10]. The aim of the present thesis was to evaluate the functional and mechanistic properties of selected mammalian and fish AMPs in three different settings in view of their possible development as novel anti-infective agents for human and veterinary application.

1) Mastitis caused by the yeast-like algae of the genus *Prototheca*.

The yeast-like algae of the genus *Prototheca* are ubiquitous saprophytes causing infections in immunocompromised patients and granulomatous mastitis in dairy cattle. Importantly, the latter infection is responsible for heavy economic losses due to dramatic loss in milk yield and its quality, as well as culling of the infected cows.

Presently, novel anti-*Prototheca* agents are necessary due to the increasing incidence of infection in dairy herds worldwide [120-122] and to the rapid spread of *Prototheca* isolates resistant to the few currently available antimicrobial drugs [121,123]. In this regard, AMPs may be promising candidates as they rapidly kill a broad spectrum of microbes *in vitro*, also including bacterial mastitis pathogens [124,125]. In the present study, three different peptides have been examined comparatively for their *prototheca*-inactivating properties, to provide functional and mechanistic information in view of their possible development as effective anti *prototheca* drugs. The three peptides were selected on the basis of distinct structural features that are strictly related to the membrane-binding capacity of these molecules, leading to different mechanisms of action. The antimicrobial activity of the selected AMPs was evaluated against several *prototheca* isolates by the minimum inhibitory concentration (MIC) and time killing assays. The mechanism of action was investigated by measuring the release of ATP in the medium and by Scanning

Electron Microscopy (SEM) in order to monitor the permeabilization and to observe the morphological alteration of *prototheca* cells. Moreover, the membrane binding capacity of these AMPs was evaluated by Circular dicroism (CD) studies in the presence of neutral and anionic liposomes mimicking yeast and bacterial cell membranes, respectively.

2) Female reproductive tract infection caused by *Candida spp.*

Vulvovaginal candidiasis affects approximately 75% of fertile age women with 5-10% incidence of recurrent infection. [126]. Importantly, the ability of *Candida spp* to form stable biofilms often results in serious medical device-related infections. Given the growing threat of drug resistance to the most common treatments, there is a great demand for novel anti *Candida* agents. In this regard, AMPs may be promising candidates for the development of novel antiinfective agents due to their broad-spectrum antimicrobial activity also against bacterial and fungal biofilms.

The aim of this study was to investigate the *in vitro* efficacy of two cathelicidins (BMAP-28 and LL-37) against planktonic and sessile *Candida* cells, in comparison with miconazole (MCZ) and amphotericin B (AMB). The study included the reference strain *C.albicans* SC5314 and a total of 24 vaginal isolates of *Candida spp*. Minimum inhibitory (MICs) and minimum fungicidal concentrations (MFCs) for planktonic cells were determined in standard conditions and in synthetic vaginal simulated fluid at vaginal pH values. The activity of the compounds was further tested against sessile *Candida spp*. by fluorescence microscopy and XTT reduction assays to quantify the inhibitory effect on biofilm cell viability. *Candida* biofilms were grown on standard polystyrene microtiter plates and on uncoated and peptide-coated medical grade silicone surfaces.

3) Prevention of fish infections in aquaculture.

Aquaculture supplies around 50% of fish needed globally for human consumption and is currently the fastest growing animal food producing industry, with a 6-8% annual rate growth [127]. However, the occurrence of infectious diseases is a major concern for fish farmers and is often the most significant cause of economic

losses [128]. There is a growing need to find new approaches for the prevention of infectious diseases of cultured fish.

Like other organisms, fish produce different kinds of antimicrobial peptides (AMPs), which are involved in host defense mechanisms. At difference with mammalian species, little is known about the immunomodulatory effects of these peptides in fish. Improving the knowledge of these effects could be useful in view of developing AMPs as immunostimulants to prevent aquaculture infections.

In this regard, In this part of my thesis, I analyzed the effects of a salmonid cathelicidin in comparison with the bovine cathelicidin BMAP-28 on the respiratory burst and on the expression of pro-inflammatory cytokine genes in primary head kidney leukocytes from rainbow trout, by using a luminometric method and RT-qPCR analysis, respectively. Moreover, the cytotoxicity of these peptides on primary head kidney leukocytes and a the RTG-2 cell line was evaluated by measuring the release of lactate dehydrogenase in the medium.

3 MASTITIS CAUSED BY THE YEAST-LIKE ALGAE OF THE GENUS *Prototheca*

3.1 INTRODUCTION:

3.1.1 The genus *Prototheca*

The genus *Prototheca*, which was thought to be a fungus, is now generally considered to be the apochlorotic equivalent of the algae *Chlorella* and consists of unicellular, aerobic, achlorophyllous, saprophytic algae with a wide distribution in natural environment. They have been isolated from environmental reservoirs such as food, milk, soil and above all sewage water and animal waste [129]. They have been shown to transiently colonize human skin, fingernails, respiratory tract and digestive system. *Prototheca* cells are ovoid to spherical in shape and range from 3 to 30 μm in diameter depending on the species and stage of life cycle [130]. These algae reproduce asexually by multiple fission, giving rise to mother cells (sporangia) from which a variable number of endospores are passively released upon rupture of the sporangium (Figure 3.1). They have a small, centrally situated nucleus, cytoplasm with abundant ribosomes, golgi body, endoplasmic reticulum, mitochondria and two-layered cell wall composed by a thin outer layer and a much thicker inner translucent layer [131]. It is worth to mention that the presence of this two-layered cell wall and the absence of chloroplast are one of the most distinctive hallmarks of *prototheca* spp compared to *chlorella* algae [132]. Regarding nutritional traits, all *prototheca* spp utilize ammonium salt and assimilate glucose and fructose whereas disaccharides are not metabolized. The taxonomy position of *Prototheca* has been disputed for a long time. Owing to their ultrastructure, the occurrence of plastid-like granules in plasma, their asexual method of reproduction, they are nowadays classified among the lower algae, the *Chlorophyceae* [133]. Five species of *Prototheca* are currently assigned to the genus: *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca stagnora*, *Prototheca ulmea* and *Prototheca moriformis*. Among these, only *P. zopfii* and *P. wickerhamii* are recognized to be pathogenic for both humans and animals [129]. Several phenotypic studies support the identification of distinct clusters within *Prototheca*, which have

been named “variants” [133]. P. *zopfii* has consistently been divided into three variants or biotypes (I to III) based on biochemical and serological differences. For example, *P. zopffi* biotype I, contrary to *P zopfii* biotype II, was able to strongly assimilate galactose and glycerol.

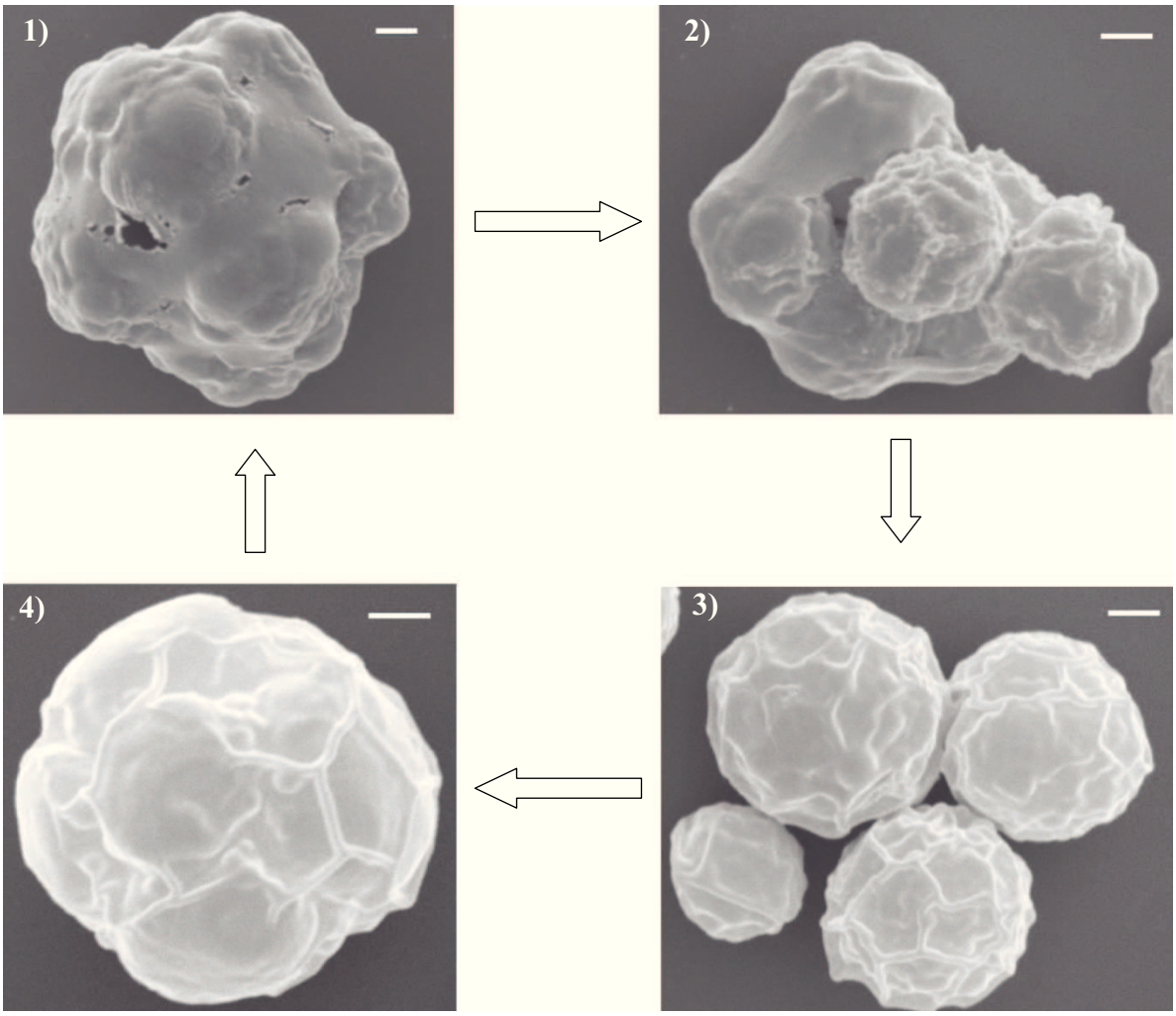

Figure 3.1 *Prototheca wickerhamii* life cycle. 1) Rupture of the sporangium. 2) Passive release of endospores from the broken sporangium. 3) Endospores. 4) Sporangium.Scanning Electron Microscopy (SEM). Magnification, x15 000; bars, 1 μm.

3.1.2 Pathogenesis and clinical manifestation

Protothecal infections are exogenous and frequently develop through contact with contaminated water. A portal for internal inoculation may by provided by trauma, surgery and in some cases by insect bites, since insects populate the slime flux of trees. Although neither animal-to-human nor human-to-human transmission has been proven [129] animal-to-animal trasmission has been observed in the cases of

prototheca mastitis in dairy herds [134]. Moreover, it has been suggested that the incubation period for *Prototheca* infection could range from weeks to months [135].

3.1.2.1 Human protothecosis

Protothecosis occur globally and has been reported on every continent except Antartica [132]. Cases of human infections have been reported from Europe, Asia, Africa, Oceania, United States, particularly the southeast states [130]. The occurrence of protothecosis can be local or disseminated and acute or chronic, with the latter being more common. Human protothecosis are mainly caused by *Prototheca wickerhamii* and have been classified in three clinical forms: cutaneous lesions, olecranon bursitis and disseminated or systemic infections [129].

The majority of cutaneous lesions have been related to patients with severe underlying disease or different forms of immunosuppression, including AIDS, renal transplantation, and lupus erythematosus. However, cases of cutaneous protothecosis have also been reported in patients with an intact immune system [130]. The most common presentation of cutaneous protothecosis is usually a vesiculobullous and ulcerative lesion with purulent discharge and crusting although other forms of cutaneous lesion have been reported including erythematous plaques, nodules, papules, herpetiform and superficially ulcerative lesions [136,137].

The reason for the predilection for the olecranon bursa as a site of *Prototheca* infection is unclear but may reflect the predisposition of this area to repeated trauma. Signs and symptoms appear gradually several weeks following the trauma and include mild induration of the bursa accompanied by tenderness, erythema, and production of variable amounts of serosanguinous fluid [130].

There are relatively few documented cases of systemic forms of protothecosis. Moreover, *Prototheca* have been isolated in connection with various illnesses such as meningitis, endocarditis and peritonitis. As in cutaneous infections, in most of these reports a number of predisposing factors, such as cancer, AIDS, organ transplantation, steroid use, likewise dialysis, catheterization and prolonged endotracheal intubation have been described. [129].

Although human protothecosis is extremely rare it should be considered in the differential diagnosis of unusual infections, especially in patients who are severely immunocompromised [130].

3.1.2.2 Animal protothecosis

Aside from causing protothecosis in humans, *Prototheca* spp are able to cause infections also in animals. In this regard, mammary gland infections (mastitis) were observed in dairy herds [138,139], cutaneous, ocular, enteric and systemic infections in dogs [140], renal granulomatous disease in salmon parr [141], and systemic infections in mice, rats and pigs [142,143]. Among these infections, mastitis is the most common form of protothecosis in animals. Mastitis, that nowadays continues to be the most frequent and expensive disease of dairy cows, is usually a consequence of microbial infection caused by pathogens that find their way into the lumen of the gland through the teat canal [144]. About 150 species of microorganisms were found as etiological agents of mastitis. Apart of different species of bacteria, several other groups of microorganisms such as yeast, fungi and algae from *Prototheca* genus can cause an inflammatory process and alterations in the udder [145]. Mastitis in cows is mostly caused by *Prototheca zopfii* and is often recognized as a chronic and symptom-less process. Importantly, this disease is responsible for heavy economic losses due to dramatic loss in milk yield and its quality, as well as culling of the infected cows. [146]. *Prototheca spp*. are reported to be sensitive to amphotericin B (AMB) and variably susceptible to azoles and other drugs *in vitro* [129,130]. However, the *in vivo* efficacy of these drugs in mastitis treatment is unpredictable, and the only completely effective control measure to date is culling of infected animals [147].

3.1.3 AMPs as anti-*prototheca* agents

Novel agents effective against *Prototheca* are necessary due to the increasing incidence of infection in dairy herds worldwide [120,122] and to the rapid spread of *Prototheca* isolates resistant to the few currently available antimicrobial drugs [123]. In this regard, AMPs may be promising candidates for the development of such drugs as they rapidly kill a broad spectrum of microbes *in vitro*, also including bacterial mastitis pathogens [124,125]. The involvement of AMPs in udder infection is in any case suggested by the observation that some of them are produced by bovine mammary gland epithelial cells [148,149] as well as by activated neutrophils recruited during infection [124] and are secreted from these cells into bovine milk [150]. At least seven bovine cathelicidin peptides belonging to several distinct structural classes [28] and 26 different bovine defensins or the genes encoding them have been reported. In the

present study, two bovine cathelicidin peptides, bovine myeloid antimicrobial peptide (BMAP-28) and bactenecin 5 (Bac5), and the β-defensin lingual antimicrobial peptide (LAP) have been examined comparatively for their *Prototheca*-inactivating properties. The three peptides were selected on the basis of distinct structural features, i.e., BMAP-28 is a linear, amphipathic α-helical peptide, Bac5 likely has an extended poly-L-proline type II conformation, and LAP has a β-sheet core stabilized by three disulfide bridges. The three peptides were selected on the basis of distinct structural features that are strictly related to the membrane-binding capacity of these molecules, leading to different mechanisms of action.

3.2 MATERIALS AND METHODS:

3.2.1 Peptides

Solid phase synthesis of BMAP-28 and Bac5 (Table 3.1), using Fmoc-chemistry, has been described previously . The β-defensin LAP (Table 3.1) was synthesized with a microwaveassisted Liberty synthesizer (CEM Corp., Matthews, NC, USA) on Lys(Boc)-substituted Tentagel resin (substitution 0.16 meq/g, 0.05 mmol scale, Novabiochem, UK) essentially as described previously [151,152]. The good quality of the crude, fully reduced peptide (MW 4520.4) as verified by electrospray ionization-mass spectrometry (ESI-MS) (Esquire 4000, Bruker Daltonics Inc., Billerica, MA, USA) allowed oxidative folding without prior purification, as described previously [151]. Complete oxidation was confirmed by ESI-MS of the peptide (MW 4514.4), whereas the correct connectivity was partly established by ESI-MS analysis of the tryptic digest, in which the disulfide-linked fragments GICVPIR and QIGTCLGAQVK (MW 1870.9) confirmed the presence of the Cys^2–Cys^4 bridge. Peptide concentrations were determined in aqueous solution by measuring the absorbance at 257 or at 280 nm, taking into account the extinction coefficients of Trp and Tyr (6839 cm^{-1} M^{-1} at 280 nm) for BMAP-28 and Tyr (1280 cm^{-1} M^{-1} at 280 nm) for Bac5. The concentration of LAP was determined using the Waddell method [153].

3.2.2 Microbial Strains and Growth Conditions

All the isolates were collected from cases of clinical and subclinical mastitis in dairy herds from Lombardia region (Italy) and included 12 strains of P. zopfii, three strains of *Streptococcus agalactiae* and five strains each of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus uberis*. The *Prototheca* isolates were identified as *P. zopfii* biotype II on the basis of biochemical features, essentially as described previously [154]. *P. wickerhamii* ATCC 16529 was the reference strain. The algae were grown on Sabouraud dextrose agar (Oxoid, UK) at 30 °C for 3–4 days, maintained in a refrigerator and transferred once a month to fresh medium. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. epidermidis* were maintained in Luria–Bertani agar plates (Oxoid), *S. uberis* and *S. agalactiae* in blood agar plates (Oxoid).

3.2.3 Antimicrobial Assays

Prototheca zopfii and *P. wickerhamii* were refreshed by streaking a loopful of algal cells onto Sabouraud dextrose agar. After incubation at 30°C for 3 days, algae were harvested from the plates and resuspended in Sabouraud medium. The bacteria were cultured in Mueller–Hinton (Difco, Detroit, MI, USA) or brain heart infusion broth (Difco) (*S. uberis*) for 18 h, diluted 1:50 in fresh medium and allowed to grow in a shaker at 37°C. Mid-log-phase bacteria were harvested after 10 min of centrifugation at 1000g and resuspended in appropriate media. The density of microbial suspensions was assessed by measuring the turbidity at 600 nm, with reference to previously determined standards, and adjusted to give $1*10^5$ and $5*10^5$ colony forming units (CFU)/ml for *Prototheca* and bacteria, respectively. The minimum inhibitory concentration (MIC) was determined by a broth microdilution assay in 96-well microtiter plates, essentially as described [75]. For *P. wickerhamii*, after the determination of the MIC, 50 μl aliquots of the assay mixture were plated on Sabouraud dextrose agar plates and incubated for 3 days at 30°C in order to determine the minimum fungicidal concentration (MFC). The microbicidal activity against *P. wickerhamii* ATCC 16529 was assayed by incubating cells ($1*10^5$ CFU/ml) at 30°C in the presence of different concentrations of each peptide in Sabouraud. At fixed time points, culture samples were serially diluted in ice-cold physiological salt solution, and 50 μl aliquots were plated onto solid Sabouraud. After 3 days of incubation at 30°C, colonies were counted and the CFU per millilitre calculated. The concentration of peptide that inhibited the growth of half of an inoculum of P. wickerhamii (IC_{50}) was estimated from dose–response curves, using the non-linear regression fit function of GRAPHPAD PRISM version 5.0 (GraphPad Software Inc., San Diego, CA, USA)

3.2.4 ATP Bioluminescence Assay

Prototheca wickerhamii ATCC 16529 cells ($1*10^5$ CFU/ml) were incubated at 30°C for 10–60 min with each peptide in Sabouraud medium. Cells were then centrifuged (1,000 g, 10 min) and the supernatants placed on ice until assayed for extracellular adenosine triphosphate (ATP). Cell pellets were submerged in liquid nitrogen for 5 min and resuspended in boiling water. Samples were then boiled for 5 min, centrifuged and the supernatants placed on ice until assayed for intracellular ATP. ATP levels were determined by luminometry using a luciferin–luciferase assay kit

(Invitrogen, Carlsbad, CA, USA), and the amount of extracellular ATP was expressed as percentage of the total cellular ATP (extracellular + intracellular).

3.2.5 Preparation of Liposomes

Large unilamellar vesicles (LUVs) were prepared by extrusion of anionic phosphatidyl/diphosphatidyl-DL-glycerol [PG/dPG, 95:5 (w/w)] from egg yolk lecithin and bovine heart, respectively. Zwitterionic LUVs were prepared using neutral L-a-phosphatidylcholine and sphingomyelin from egg yolk and ergosterol dispersions [PC/SM/Er, 40:40:20 (w/w)]. All components were purchased from Sigma-Aldrich. Dry lipids were dissolved in chloroform, evaporated under a stream of nitrogen, and the residue was vacuum-dried for 3 h. The lipid cake was resuspended to a concentration of 3mg/ml in the appropriate buffer by spinning the flask at a temperature above the lipid critical temperature. The resulting multilamellar vesicle suspensions were disrupted by several freeze–thaw cycles prior to extrusion with a miniextruder (Avanti Polar Lipids Inc., Alabaster, AL, USA) through polycarbonate filters with 100 nm pores. For all experiments, LUVs were freshly prepared or used within 1 or 2 days.

3.2.6 Circular Dichroism

Circular dichroism (CD) spectroscopy was performed on a J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA), using 2-mm quartz cells and 20 μM peptide with phospholipid vesicles in phosphate-buffered saline (PBS, 0.15M NaCl, 0.01M sodium phosphate, pH 7.0) at room temperature (25°C). Peptide/lipid suspensions (molar ratio 1:20) were incubated for 30 min at 37°C before use. These spectra were compared with those obtained in the absence of LUVs (aqueous buffer), in the presence of trifluoroethanol (TFE), a solvent known to stabilize ordered conformations, and in the presence of anionic LUVs composed of PG/dPG that mimic bacterial membranes. Spectra were the average of at least two independent experiments, each with an accumulation of three scans.

3.2.7 Scanning Electron Microscopy

About $1*10^7$ CFU/ml of *P. wickerhamii* ATCC 16529 were incubated at 30°C in Sabouraud medium with each peptide. After incubation for 1 or 6 h at a peptide concentration (40 μM) that ensured 70–90% killing by all peptides, 15 μl of cell suspensions were deposited onto polylysine-coated glass coverslips and fixed at 4°C

overnight with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer, pH 7.3. The coverslips were then extensively washed with PBS, post-fixed at 4°C for 1h with 1% (v/v) osmium tetroxide in PBS and dehydrated in graded ethanol solutions. After lyophilisation and gold coating, the samples were examined on a Leica Stereoscan 430i instrument (Leica Inc., Deerfield, IL, USA).

3.2.8 Statistical Analysis

Statistical differences among groups of data were analysed by one-way analysis of variance followed by the Bonferroni post test, using GRAPHPAD Prism version 5.0. In all comparisons, $P<0.05$ was considered significant.

3.3 RESULTS:

3.3.1 Antimicrobial and Permeabilizing Activity

The antimicrobial activity of BMAP-28, Bac5 and LAP (amino acid sequences reported in Table 3.1) was assayed against various microbial strains isolated from cases of bovine mastitis, including nonphotosynthetic algae of the genus *Prototheca* and a number of contagious (*S. aureus* and *S. agalactiae*) and environmental (*S. uberis*, *S. epidermidis*, *E. coli* and *K. pneumoniae*) bacterial species. In the standard microdilution assay, BMAP-28 was effective in the low micromolar range against all strains (Table 3.2), with the exception of a single *S. uberis* isolate that was resistant to this peptide up to the highest concentration tested (32 μM). Bac5 was as effective as or even better than BMAP-28 against *Prototheca* spp. This AMP was also very effective against Gram-negative organisms while displaying high MIC values against most Gram-positive organisms, particularly *S. aureus* and *S. uberis*, in line with the reported selectivity of Pro-rich AMPs for Gram-negative species [155]. Under the same assay conditions, the β-defensin LAP inhibited the growth of *Prototheca* spp. with an average MIC value of 8 μM but was ineffective against all bacterial isolates even at the highest concentration tested (32 μM) (Table 3.2). LAP did however display a significant antibacterial activity when assayed in fourfold diluted bacterial growth media (Table 3.2, values in parentheses). These results are in agreement with the widely reported salt and medium sensitivity of the antibacterial activity of β-defensins [156]. whereas the anti-algal activity appears to be more robust. The MIC data reported in Figure 3.1 indicate that all three peptides are effective against all *Prototheca* isolates.

Table 3.1 Sequences of bovine antimicrobial peptides

Pepide	Sequence	MW	*q*[a]	%H[b]
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI-NH2	3074	+8	44
Bac5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP-NH2	5147	+10	30
LAP	GVRNSQS**C**RRNKGI**C**VPIR**C**PGSMRQIGT**C**LGAQVK**CC**RRK[c]	4514	+10	37

[a] *q*, charge.

[b] %H, percent of hydrophobic residues (A,V, M, L, I, F, Y, W). Cysteine residues are considered to be hydrophobic

[c] Cysteine residues involved in disulfide bridge formation are in bold. The cysteine arrangement is 1-5, 2-4, 3-6.

Table 3.2 Antimicrobial activity of bovine peptides against isolates from cases of mastitis

	MIC range (μM)		
Organism (no. of tested strains)	**BMAP-28**	**Bac5**	**LAP**
***Prototheca* spp. (12)** [a]	**2-8**	**0.5-2**	**4-16**
***Escherichia coli* (5)** [b]	**4 – 8**	**0.5 - 1**	**32 (8)**[c]
***Klebsiella pneumoniae* (5)** [b]	**1 – 2**	**1 - 4**	**>32 (32)**[c]
***Staphylococcus aureus* (5)** [b]	**2 – 4**	**>32**	**>32 (4)**[c]
***Staphylococcus epidermidis* (5)** [b]	**1 – 2**	**1 - 2**	**32 (1)**[c]
***Streptococcus agalactiae* (3)** [b]	**2**	**4 - 16**	**>32**
***Streptococcus uberis* (5)** [d]	**2 - >32**	**16 - >32**	**>32**

[a] determined in Sabouraud broth

[b] determined in 100% Mueller-Hinton broth

[c] values in parentheses determined in 25% Mueller-Hinton broth

[d] determined in brain heart infusion broth

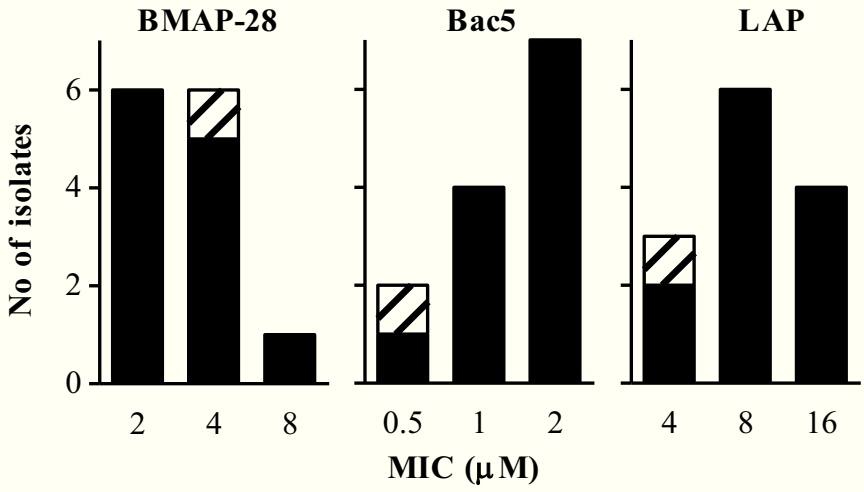

Figure 3.1 Distribution of MIC values of the indicated peptides for *Prototheca* spp. isolates. The hatched part of the bars refers to *P. wickerhamii* ATCC 16529.

An increasing number of studies indicate that this algal microorganism is an emergent mastitis pathogen refractory to antibiotic therapy [123], and particularly, *P. wickerhamii* has been shown to affect humans by producing cutaneous infections in immunocompromised patients [157]. These considerations prompted us to further investigate the antimicrobial effects of BMAP-28, Bac5 and LAP against *P. wickerhamii* ATCC 16529 as a reference strain. Its growth was inhibited by the AMPs with MIC values ranging from 0.5 μM (Bac5) to 4 μM (BMAP-28 and LAP), as compared with a MIC of 0.2 μM for AMB (Table 3.3), which has been already reported to be active against this strain [130]. In a microbicidal activity assay, BMAP-28 was found to decrease the viability *of P. wickerhamii* by approximately 80% in only 10 min (Figure 3.2A) and by >95% within 60 min at a peptide concentration corresponding to its MIC value (4 μM) (Figure 3.2A and B). The microbicidal effects of LAP and Bac5 were instead significantly slower. At their MIC concentration, they were not effective against *P. wickerhamii* up to 30 min and caused an approximately 20% decrease in cell viability within 60 min (Figure 3.2A). Accordingly, the IC_{50} calculated following 10 and 60 min incubation was considerably higher for Bac5 and LAP than for BMAP-28 (Table 3.3). Increasing the concentration of Bac5 and LAP to16 μM resulted in 60% and 40% killing activity, respectively, following 60 min cell incubation (Figure 3.2B), whereas complete killing was only achieved following 6 h incubation of algal cells with each peptide at its MFC value, corresponding in both cases to four times the MIC value (i.e., 2 μM Bac5 and 16 μM LAP) (Figure 3.2A). Importantly, none of the peptides was cytotoxic for BME-UV1 cells, used as a model for bovine mammary gland epithelium [158], at its algicidal concentration (cell viability higher than 95% as assessed by MTT assay). The viability of these cells was only slightly affected by BMAP-28 (approximately 80% viable cells) at fourfold its algicidal concentration, whereas Bac5 and LAP were ineffective at corresponding concentrations. Overall, these data indicate a much lower affinity of these peptides for BME-UV1 as compared with *Prototheca* cells. This is in line with the results of numerous studies indicating that AMPs preferentially associate with target microorganisms rather than with host cells [82,159]. Intrinsic molecular and structural characteristics of microbial versus host cells as well as inherent structural features of AMPs are thought to be major determinants of this selective antimicrobial discrimination [159].

Table 3.3 Antimicrobial activity against *P. wickerhamii* ATCC 16529

Test agent	MIC (µM)	MFC (µM)	IC_{50} (µM)	
			10 min	60 min
BMAP-28	4	4	2	1
Bac5	0.5	2	6	2
LAP	4	16	32	16
AMB	0.2	> 1.6	-	-

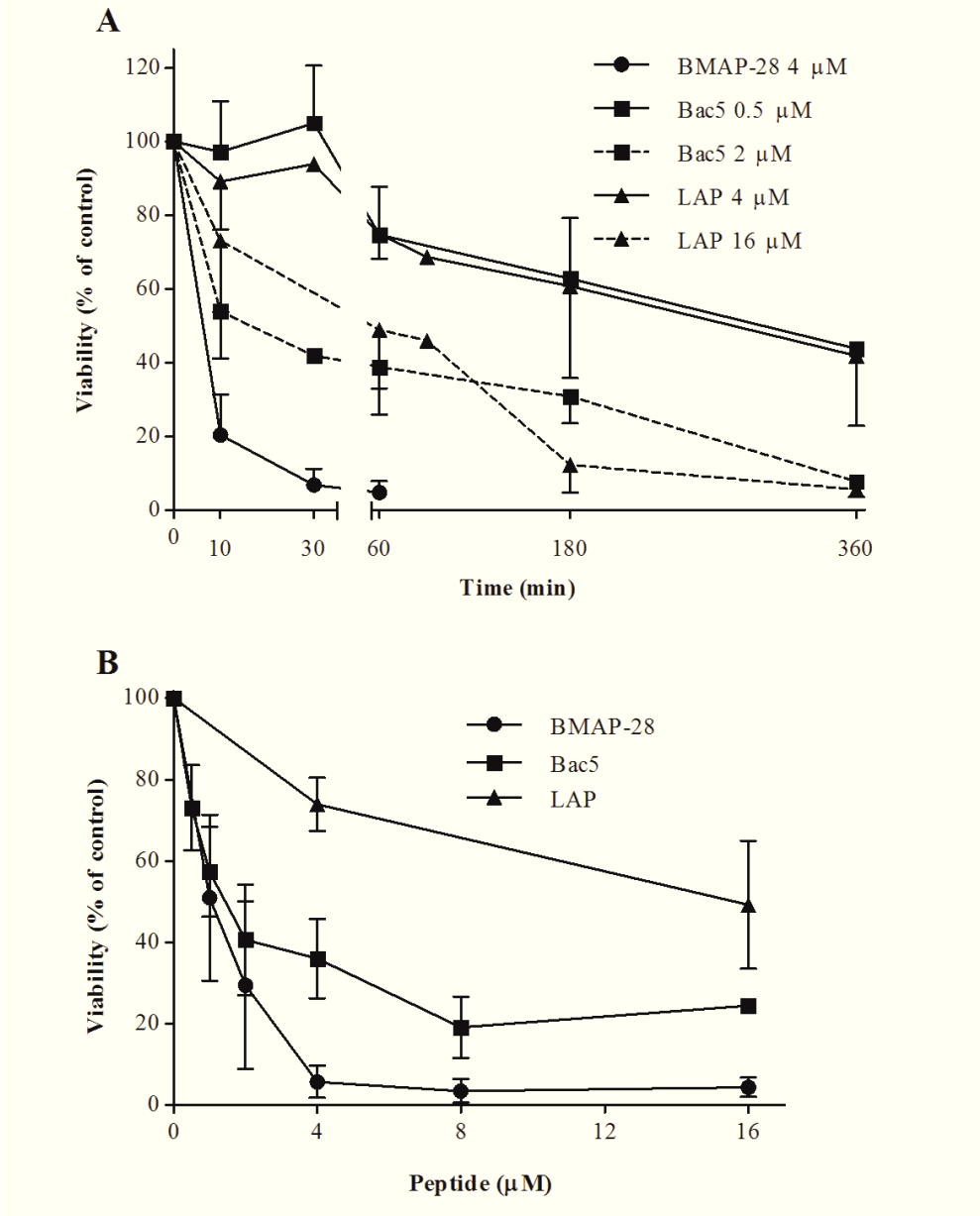

Figure 3.2 Time-killing curve (A) and dose–response after 60 min incubation (B) of BMAP-28, Bac5 and LAP against *P. wickerhamii* ATCC 16529. Cells were incubated with peptides at the indicated concentrations in Sabouraud medium. At selected time points, samples were serially diluted and plated to allow colony counts. The results are reported as percent viability relative to untreated cells and are the mean ± SD of at least three independent experiments.

To gain further insight into the mechanism of antimicrobial action, peptides were analysed for their capacity to induce extracellular release of ATP from *P. wickerhamii* as a means to evaluate cell membrane perturbation. At its MIC value, BMAP-28 induced, respectively, 80% and 100% release of cellular ATP after 10 and 60 min exposure (Figure 3.3), consistent with a rapid membranolytic activity. In contrast, Bac5 and LAP did not promote ATP release for up to 60 min incubation at their MIC values. This was confirmed by a lack of propidium iodide uptake under the same experimental conditions (not shown). Approximately 50% ATP release was observed after cell incubation with Bac5 at fourfold MIC value. Under the same concentration conditions, LAP caused approximately 25% permeabilization (Figure 3.3).

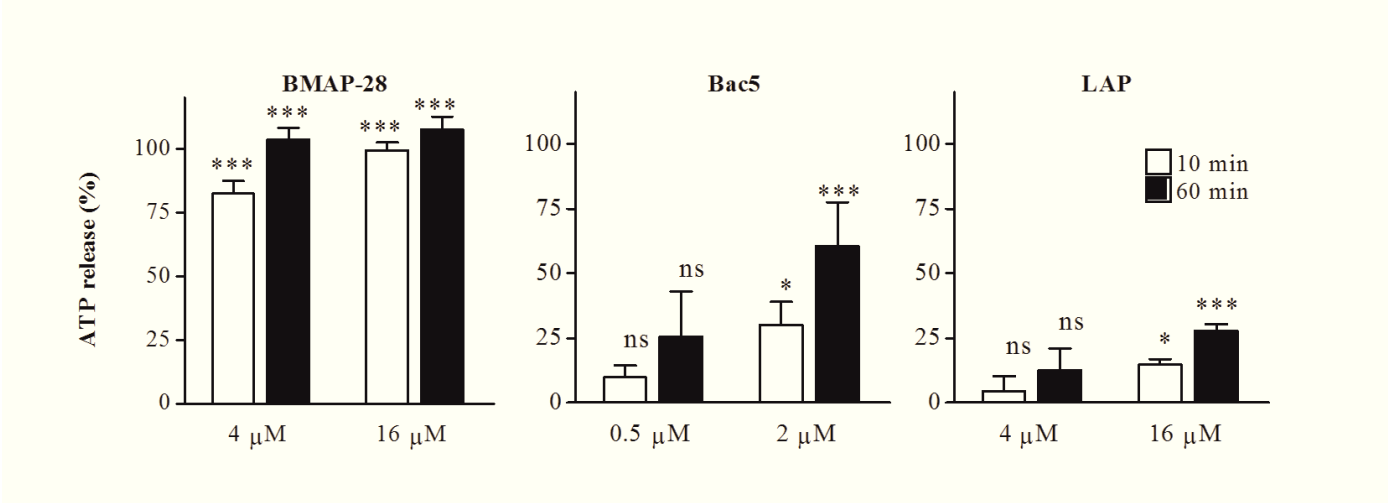

Figure 3.3 ATP release from peptide-treated *Prototheca* cells. Cells were incubated for 10 and 60 min with the indicated peptides at MIC and fourfold MIC values. The amount of ATP in the cell supernatants was expressed as percentage of the total cellular ATP. Results are the mean ± SD of at least three independent experiments. *P<0.05; **P<0.01; ***P<0.001.

3.3.2 CD Spectroscopy

A different propensity of the peptides for interaction with the plasma membrane was also suggested by CD spectra determined in the presence of zwitterionic LUVs containing ergosterol, a simple model for algal cell membranes. As shown in Figure 1.4, the CD spectrum of BMAP-28 showed a strong conformational transition from random coil in aqueous buffer to a-helix in the presence of 50% TFE (Figure 3.4A). Under these conditions, all BMAP-28 molecules likely have a similar helix content, which was estimated at approximately 50% [160], compatible with formation of an N-terminal helix and disordered C-terminal tail [73]. The spectrum in the presence of zwitterionic LUVs indicates a lower yet detectable helix content, which may be

explained by a smaller part of the peptide being helically structured or, more likely, only part of the peptide population being membrane bound in a helical conformation, estimated at about 20% by comparison with the TFE spectrum. The CD spectrum of BMAP-28 in the presence of anionic vesicles was considerably more intense, and the minimum shifted from about 208 to 222 nm. This indicates a stronger membrane interaction and is possibly consistent with helix aggregation at the membrane (as suggested by the altered $\theta_{208}/\theta_{222}$ ratio. [161]) and/or increased content of β-structure, presumably at the C-terminus of the sequence (Figure 3.4A). Bac5 showed similar spectra in aqueous buffer and in the presence of neutral LUVs (Figure 3.4B), indicating that its conformation was not markedly altered in their presence. The spectra are consistent with an extended type II poly-L-proline conformation, in accordance with previous reports [162,163]. Similar slight changes in the shape of the CD spectrum were observed in the presence of TFE or anionic LUVs. Taken together, these results indicate that Bac5 does not markedly alter its conformation in the presence of biological membranes and are more consistent with a surface interaction (especially with anionic LUVs where it is favoured by electrostatic attraction) than membrane insertion as for BMAP-28. The CD spectrum profile of LAP measured in aqueous solution was consistent with a partly β-sheet, partly random coil conformation (Figure 3.4C) and was unaffected in the presence of zwitterionic LUVs, indicating poor interaction of the peptide with this type of membrane. A helical component became evident in the presence of TFE or of anionic vesicles (Figure 3.4C), likely due to structuring of the N-terminal segment, as also observed for hBD3 under similar conditions, and may result from some form of interaction with bacterial-type membranes [164].

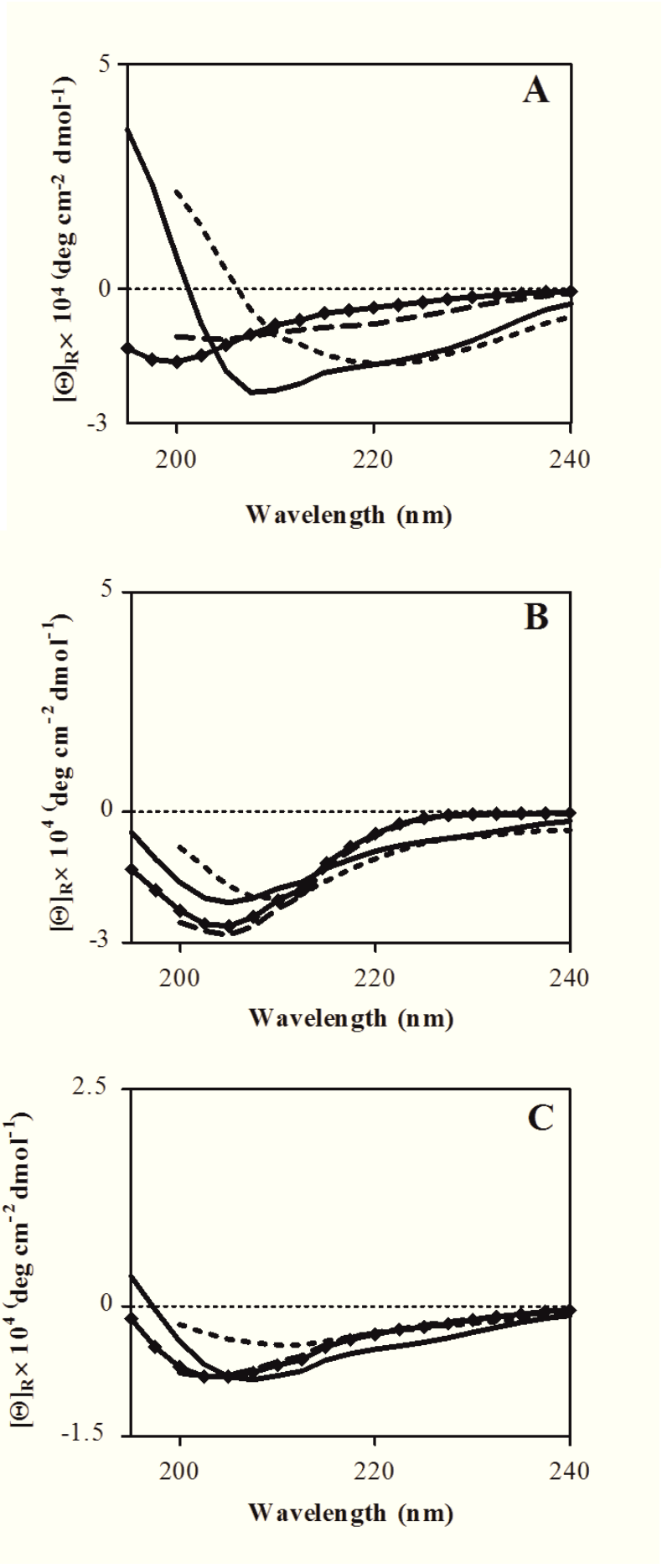

Figure 3.4 CD spectra of BMAP-28 (A), Bac5 (B) and LAP (C) in PBS (diamonds), 50% TFE (solid line), PG/dPG (short dashes) and PC/SM/Er (long dashes) LUVs. The concentrations of peptide and lipid in PBS were 20 and 400 mM, respectively.

3.3.3 SEM Analysis of Surface Alterations

To further investigate the mechanism of action, we used scanning electron microscopy (SEM) to visualize morphological alterations induced by each peptide, which could be related to its killing mechanism(s). Representative micrographs of untreated *P. wickerhamii* showed individual cells with ridged surface (Figure 3.5A) or morula-like structures (Figure 3.5B) typical of *P. wickerhamii* sporangia. As shown in Figure 3.5C and D, incubation for 60 min with BMAP-28 resulted in extensive surface blebbing on both individual and sporangial cells, with occasional release of intracellular material (Figure 3.5C). These features support a membranolytic killing mechanism. LAP-treated cells did not reveal evident signs of surface damage, although we consistently found that approximately 15% of treated cells had an uncharacteristic smooth, unridged surface (also showing some surface blebbing), as compared with only about 2% of untreated control cells showing this feature (Figure 3.5E and F). Cell incubation with Bac5 did not produce any visible surface alterations, even after a 6-h incubation time (not shown).

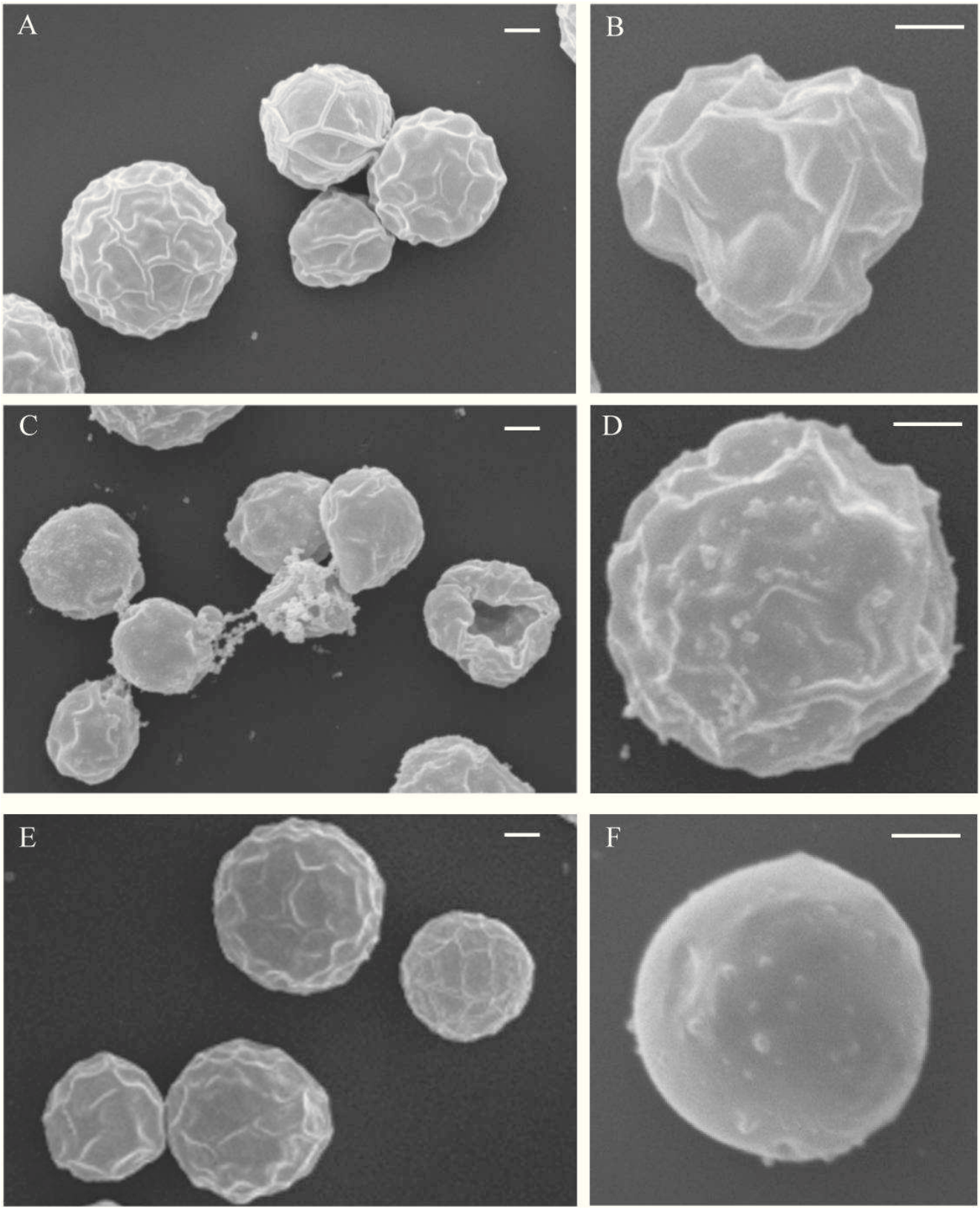

Figure 3.5. Scanning electron microscopy of *P. wickerhamii* cells incubated for 60 min in the absence (A, B) or in the presence of 40 μM BMAP-28 (C, D) or 40 μM LAP (E, F). Magnification, x15 000; bars, 1 μm

3.4 DISCUSSION:

The efficacy of currently available pharmacological therapies for the treatment of *Prototheca* infection is controversial [121,130] and is in any case undermined by the emergence of resistant strains worldwide to the few drugs in use [123]. Naturally occurring AMPs may be a promising source for the development of effective anti-prototheca agents, as these peptides generally show a rapid and broad-spectrum antimicrobial activity and are largely unaffected by resistance mechanisms to clinically used antiinfective agents [60,165]. In this regard, it has been reported that peptides derived from bovine lactoferrin, a multifunctional protein endowed with antimicrobial activity [166], show the ability to inactivate *P. zopfii* with MIC value of 1 μg/ml, although the mechanism underlying this activity has not yet been elucidated [167]. In this study, we have examined the anti-prototheca activity of chemically synthesized versions of the endogenous bovine AMPs BMAP-28, Bac5 and LAP. These peptides are highly cationic (+8 to +10), with one out of every three/four residues being positively charged, and have a variable content of hydrophobic amino acids, ranging from 30% in Bac5 to almost 50% in BMAP-28. The latter is a linear peptide with a propensity for adopting an a-helical conformation in the region encompassing residues 1–18, in a membrane-like environment [73]. The alternation of hydrophobic and hydrophilic residues confers a strong amphipathic character to the helix, favouring interaction with biological membranes [73]. Bac5 includes in its sequence a tandemly repeated Xaa1-Pro-Pro-Xaa2 motif, in which Xaa1 is almost invariably Arg and Xaa2 is a bulky hydrophobic residue (Ile, Phe or Leu) [168]. On the basis of CD and nuclear magnetic resonance spectroscopy, an extended poly-L-proline type II conformation has been proposed [162,163], but this or other possible conformations do not result in amphipathic structures, judging from Edmundson-type projections (www.bbcm.units.it/~tossi/HydroMcalc). LAP has the same global positive charge as Bac5 and an intermediate content of hydrophobic residues between BMAP-28 and Bac5. Mapping its residues on the known structures of the human defensins hBD2 and hBD3 and bovine defensin 12 (PDB ID: 1FD3, 1KJ6 and 1BNB respectively) indicates that it could have a roughly discoid structure which is predominantly polar, with a small hydrophobic patch on one edge. Antimicrobial assays indicate that all peptides are effective *in vitro* against mastitic isolates of *P. zopfii* and kill a reference strain *of P. wickerhamii* in a manner dependent on time and dose, at concentrations comparable

with or slightly higher than that of AMB. The observed decrease in CFU following treatment with BMAP-28 at its MIC value was quantitatively and kinetically correlated with the extent of ATP released from *P. wickerhamii*, suggesting a mainly membranolytic mode of action. A killing mechanism based on membrane perturbation is considered the norm for α-helical AMPs and underlies a broad-spectrum activity against Gram-positive and Gram-negative bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant Enterococcus faecium clinical isolates [73,75], as well as the fungi *Cryptococcus neoformans* and several *Candida spp* [61]. The proposed lytic mechanism for BMAP-28 is supported by CD spectra which indicate capacity to interact with anionic LUVs (models for bacterial membranes) and with ergosterol-containing zwitterionic liposomes (models for fungal/algal membranes). The plasma membrane of *Prototheca* is rich in zwitterionic phospholipids (>70% by weight of total phospholipids) and ergosterol (4% of total neutral lipid w/w) [169]. Our results are consistent with published evidence that BMAP-28 induces substantial calcein release from artificial liposomes with a composition mimicking that of fungal membranes and causes rapid uptake of the dye SYTOX green into the hyphae of the plant pathogen *Fusarium oxysporum* f. sp. *Vasinfectum* [170]. Overall, these results point to the plasma membrane as a primary target for the antifungal and anti-algal activity of BMAP-28. Bac5 and LAP exhibited a different killing behaviour. Whereas BMAP-28 sterilized *P. wickerhamii* cell cultures within 30 min incubation at its MIC, Bac5 and LAP led to a comparable decrease in CFU only after 3–6 h incubation, and only at their MFC, which corresponded to four times the MIC for both peptides. This, and the fact that, unlike BMAP-28, only modest amounts of ATP were released from *Prototheca* cells following up to 1 h incubation with Bac5 or LAP at MIC values, argue against a lytic killing mechanism. The amount of ATP in the supernatants of cells treated with lethal concentrations of these peptides increased at longer exposure times but was not proportional to the CFU reduction measured in parallel, so likely resulted from leakage of cell contents as a late effect of killing rather than from early membrane injury. This is also backed by CD studies, which suggest a looser interaction of Bac5 and LAP with neutral model membranes. The structure of Bac5 is also unaffected in the presence of phosphatidyl choline vesicles [162], and no calcein release was observed from this type of vesicle in the presence of synthetic fragments of Bac5 and its porcine analogue PR-39 [163,171,172]. The CD behaviour of LAP in the presence of neutral or anionic model membranes is similar to that of hBD3, whose weak

propensity to interact with neutral liposomes has also been demonstrated using high-resolution synchrotron small-angle X-ray scattering [172]. In short, the conformation of either peptide is little affected by the presence of neutral membranes, whose integrity is likewise little affected by peptide interaction. Significantly, human β-defensins have been shown to kill fungal cells without causing membrane disruption [173]. The differential killing mechanisms of the three peptides, and the fact that only BMAP-28 acts via membrane permeabilization is further supported by SEM analysis of peptide-treated cells, revealing extended surface damage only following incubation with BMAP-28, even at Bac5 and LAP concentrations that caused 70–90% killing and after prolonged incubation times. In this respect, members of both the Pro-rich and β-defensin AMP families have been variously shown to penetrate into bacterial or mammalian cells without compromising plasma membranes [28,151,174,175]. An accepted mechanistic model for the killing of Gram-negative bacteria by Pro-rich peptides involves interaction with intracellular targets following cellular internalization mediated by specific membrane transporters [155]. With respect to the antifungal activity of β-defensins, it appears that different members may act via distinct mechanisms, dependent on interaction with different cell wall components, which in turn are different from those used by neutrophil defensins or plant defensins [176], indicating they act in a quite complex framework. To our knowledge, this is the first report on the activity of AMPs against *Prototheca spp* apart from lactoferrin. We demonstrate that BMAP-28, Bac5 and LAP kill this organism with distinct potencies, killing kinetics and mode of action and may be appropriate for treatment of protothecal mastitis. Additionally, the ability of Bac5 and LAP to act via novel, nonlytic mechanisms could be useful for the identification of druggable targets and development of selective therapeutic agents. Future studies are thus warranted to better elucidate the respective mechanisms of action and allow the design of AMPs or derivatives with increased potency.

4 FEMALE REPRODUCTIVE TRACT INFECTION CAUSED BY *Candida* SPP.

4.1 INTRODUCTION:

4.1.1 *Candida spp* and related infections

Since the early 1980s, fungi have emerged as major causes of human disease, especially in immunocompromised patients and/or those hospitalized with serious underlying disease [177] *Candida* spp, and especially *Candida albicans*, are considered the most common cause of fungal infection leading to a range of non-life-threatening mucocutaneous to life-threatening invasive infections. [178]. Therefore, development of candidiasis depends on the delicate balance between the fungi and the host's immune status which determines the commensal or pathogenic relationship [179].

4.1.1.1 Mucosal infections

Mucocutaneous candidiasis are non-life-threatening infections that can be divided into nongenital candidiasis and genitourinary candidiasis. Among nongenitourinary candidiasis, oropharyngeal manifestations are the most common and usually are diagnosed in immunocompromised patients, such as human immunodeficiency virus (HIV)-infected people [180], whereas one of the most clinically important genitourinary disease is the vulvovaginal candidiasis [178]. Vulvovaginal candidiasis results from the overgrowth of various *Candida spp* that may be already present in the vagina as commensal organisms, and symptomatic infection is correlated with a high vaginal fungal burden. The majority of cases of vulvovaginal candidiasis are caused by *Candida albicans* (80%–85%) whereas the non-*albicans* species of *Candida* account for a 5%–20% of the cases. Among non-albicans species, *C. glabrata* is the predominant yeast recovered in vulvovaginal candidiasis, followed by other less frequent species including *C. parapsilosis*, *C. Krusei* and *C. tropicalis* [181].

It is estimated that vulvovaginal candidiasis affects approximately 70-75% of women at least once during their lives with a 5-10% incidence of recurrent infection, defined as four or more episodes every year [126]. Moreover, the incidence of vulvovaginal candidiasis is though to be increasing due to single-dose treatment, low-dosage azole maintenance regimens and indiscriminate use of over-the-counter antimycotics [182].

4.1.1.2 Invasive infections

In contrast to mucosal candidiasis which is highly prevalent but does not cause high mortality, systemic infections are life threatening, with mortality rates reaching up to 26–60% [183]. Systemic candidiasis results when the organisms enter the blood stream due to compromised immunity and/or use of medical devices, such as stents or intravenous catheters. Considering the number of patients diagnosed each year, *Candida spp* have emerged in the recent decades as one of the most important pathogens in sepsis, causing significant morbidity and mortality [184]. Moreover, *Candida spp* constitute the fourth leading cause of nosocomial bloodstream infections in the United States, accounting for 8% to 10% of all bloodstream infections acquired in the hospital [177].

4.1.1.3 Candida Biofilms

Many microbes in their natural environments are found in structured microbial communities called biofilms and not as free-floating (planktonic) organisms [185]. Biofilms are notoriously difficult to eliminate and are a source of many recurrent infections also due to reduced susceptibility to common antifungal agents [179]. Biofilms of *Candida spp* play a growing role in human medicine. Indeed, most manifestations of candidiasis are in fact associated with the formation of *Candida* biofilms on surfaces, and this phenotype is associated to both mucosal and systemic infection [186].

In general, biofilms are defined as structured microbial communities with a complex three-dimensional architecture characterized by a network of adherent cells connected by water channels and encapsulated within an extracellular matrix [187]. The biofilm formation proceeds through three distinct developmental phases: adhesion, growth and dispersion. (Figure 4.1).

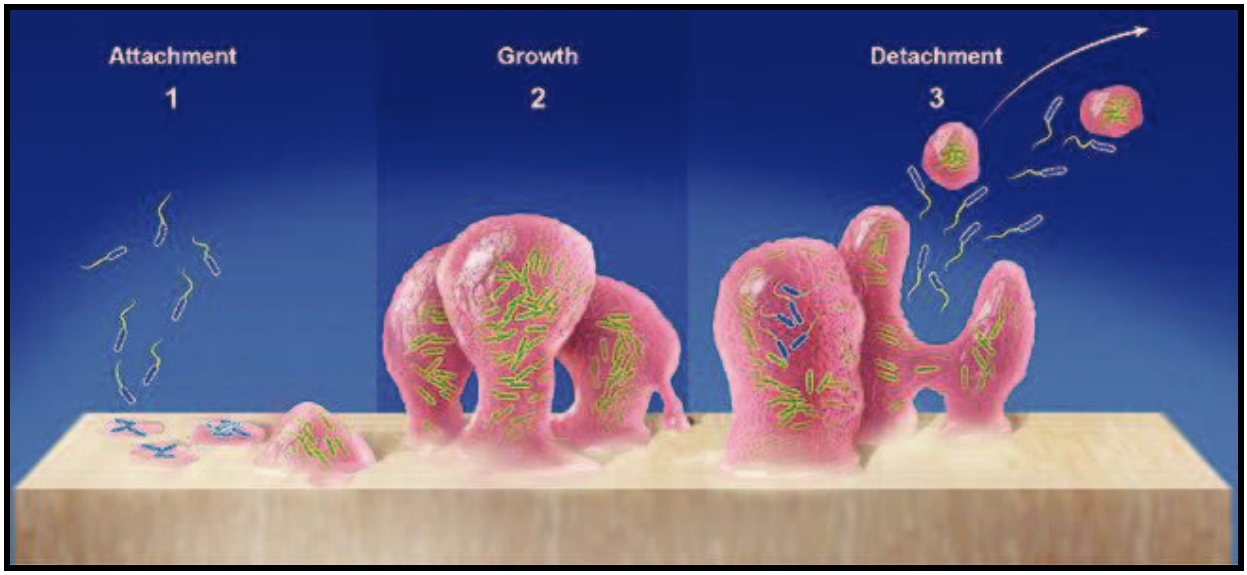

Figure 4.1 Biofilm life cycle. (Keller D and Costerton JW. 2009) [188]

Adherence of *C. albicans* cells to both inert and biological surfaces is essential for biofilm formation and is regulated by several adherence factors such as the agglutinin-like sequence (ALS) and the hyphal wall protein Hwp1 [189].

The layered architecture of biofilms observed both *in vitro* and *in vivo* suggests that the morphological transition from a unicellular budding yeast to a hyphal form plays a pivotal role in cell adhesion and the biofilm formation [190]. Indeed, hyphae have been observed to adhere and to invade host tissues more readily than the yeast form, suggesting that filamentous growth contribute to the virulence of this major human pathogen [191-193]. The ability of *Candida albicans* to switch between different morphologies is regulated by environmental conditions, such as temperature, pH or presence of chemical stimuli such as serum components or N-acetylglucosamine [194].

Biofilm formation begin with the adhesion of microbial cells to the surfaces to form microcolonies. It is during this colonization that *Candida* cells are able to communicate via quorum sensing molecules using products such as tyrosol, farnesol and farnesoic acid [195]. These quorum sensing secreted molecules are fundamental in the biofilm formation due to their pivotal role in the regulation of the expression of several genes involved in the extracellular matrix production, that, in the case of *Candida albicans*, is composed prevalently of carbohydrates together with smaller amounts of proteins [196]. The extracellular matrix is an essential and important element for a mature biofilm. Indeed, cells embedded in this matrix have significantly

different properties as compared to free-floating cells, such as the increased resistance to the antifungals, facilitated distribution of nutrients and signalling molecules and reduced metabolic activity [185].

The last step of biofilm cycle is the dispersion of biofilm colonies which can occur as a consequence of mechanical failures or can be induced by the biofilm itself in response to environmental cues such as changes in nutrient availability, fluctuations in oxygen concentration or increase in nitric oxide [197]. Biofilm dispersal is thus a naturally occurring process that represents a mechanism to escape starvation or other negative environmental conditions.

To summarize, the ability to form biofilms is intimately associated with the ability to cause infections and as such it should be considered as an important virulence factor during candidiasis. Moreover, the biofilm lifestyle results in antifungal drug resistance and protection from host defenses, both of which carry important clinical repercussions.

4.1.2 Antifungal agents currently used in therapy

Antifungal drugs currently used for the treatment of *Candida* infection include polyenes, azole, echinocandines, allylamines, flucytosine and sordarins. These drugs exert either fungicidal or fungistatic activities by interfering with essential processes (Fig 4.2) [198]. Although there are several drugs to combat fungal infection, we must take into account that the development of resistance to antifungal drugs has become increasingly apparent, especially in patients who require long-term treatment or who are receiving antifungal prophylaxis.

Polyenes bind to ergosterol in the fungal cell membrane and promote leakiness which may contribute to fungal cell death. Amphotericin B has been for many years the only antifungal polyene that could be administered systemically. Besides its potent and broad spectrum of action, the classic AMB deoxycholate (Fungizone^{TM}) formulation exerts also a considerable toxicity on mammalian cells that limit its use in therapy [199]. In an attempt to improve the therapeutic index of amphotericin B, three lipid-associated formulations were developed, including amphotericin B lipid complex, liposomal amphotericin B, and amphotericin B colloidal dispersion [200]. Amphotericin B deoxycholate showed reduced activity against *C. albicans* biofilm with respect to the activity observed against planktonic cells [201,202]. To the other hand, lipid

formulations of amphotericin B (liposomal AMB and ABLC) exhibited inhibitory activities against *C. albicans* biofilm with MICs similar to those seen for planktonic cells [203].

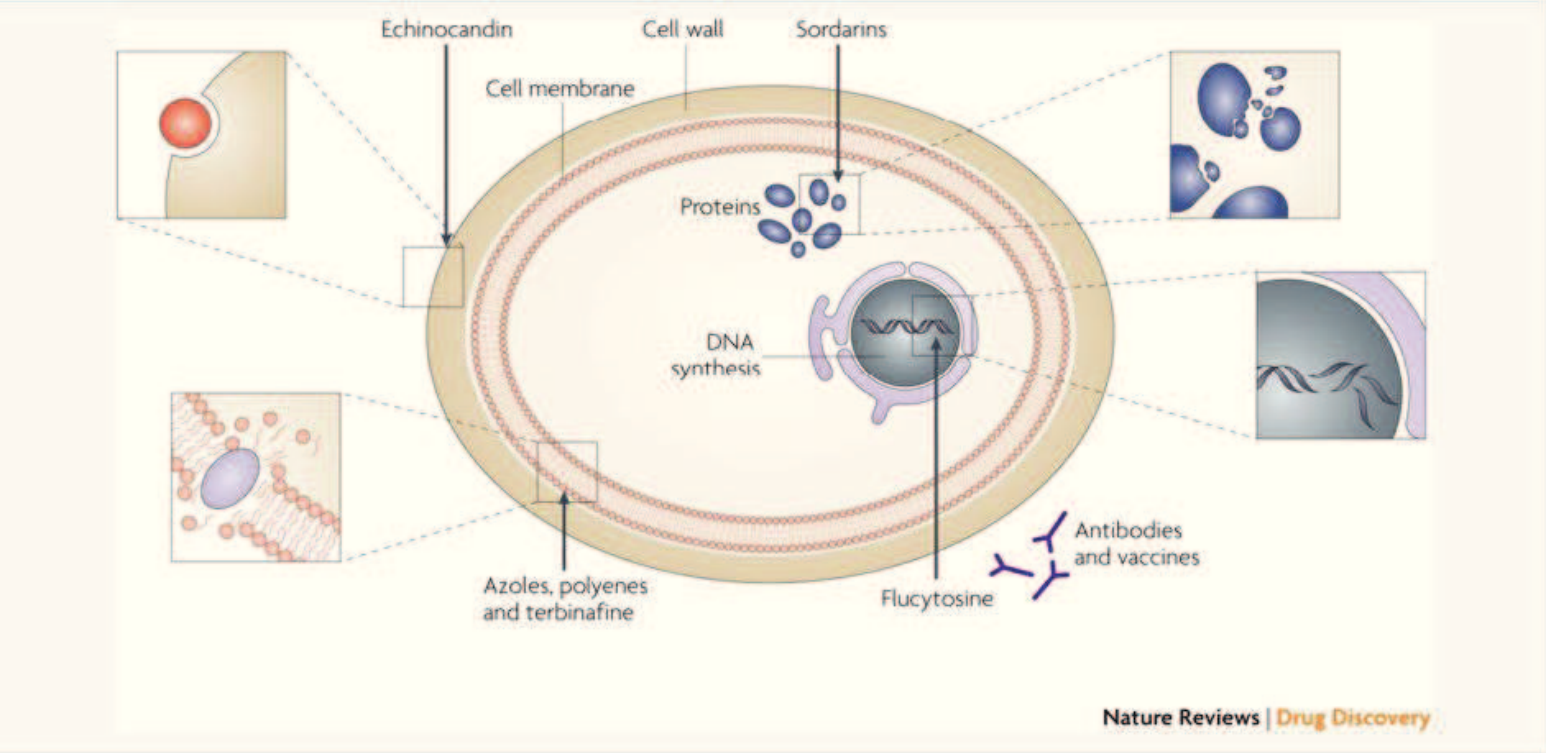

Figure 4.2 An illustration of the mechanisms of action of currently available antifungals as well as selected antifungals under development (Ostrosky-Zeichner L. et al. Nature Reviews. 2010)

For over two decades, the azole antifungals have been used in clinical practice to treat various fungal infections They are categorized into two distinctive classes: the imidazoles and the triazoles. [204]. The clinically useful imidazoles are clotrimazole, miconazole, and ketoconazole whereas two important triazoles are itraconazole and fluconazole (Figure 4.3). The generally accepted mode of action of azoles is the inhibition of 14α-lanosterol demethylase, a key enzyme in ergosterol biosynthesis, resulting in depletion of ergosterol and accumulation of toxic 14α-methylated sterols in membranes. In some yeasts, such as *Saccharomyces cerevisiae*, they can also inhibit the subsequent Δ22-desaturase step [205]. For some azoles, their antifungal mode of action is not only characterized by inhibition of ergosterol biosynthesis. In the past years it was shown that generation of reactive oxygen species is important for the antifungal activity of miconazole, pointing to an ancillary mode of action for this azole

[206]. With the exception of ketoconazole, use of the imidazoles is limited to the treatment of superficial mycoses, whereas the triazoles have a broad range of applications in the treatment of both superficial and systemic candidal infections [207]. *Candida albicans* biofilms are highly resistant to the popular azoles: not only to fluconazole, but also to the new triazoles voriconazole, ravuconazole and posaconazole. The mechanisms of azole resistance of *C. albicans* biofilms are developmental-phase specific. While efflux pumps play a critical role in the early-phase biofilms, alterations in sterol composition with reduced ergosterol levels is an important mechanism at the intermediate or mature phases of biofilm formation [208]. Resistance to fluconazole has been proven to increase up to >1000 times in *in vitro*-grown biofilms compared to planktonic cells [186].

The allylamines are a class of antifungal drugs that inhibit ergosterol synthesis at the level of squalene epoxidase. These agents are highly selective for the fungal enzyme and have a minimal effect on mammalian cholesterol synthesis. Naftifine, the original member of the allylamine series, possesses only topical activity, whereas the naftifine analog terbinafine is active both topically and systemically. *In vitro*, terbinafine is exceptionally active against dermatophytes, molds, and dimorphic fungi in which it exerts a fungicidal action also against some azole-resistant *C. albicans* strains. [209] is available as a prescription and in Over-The-Counter creams [209]. It is not recommended for use in patients with chronic or active liver disease, and patients' liver enzymes should be monitored before beginning treatment, even in those without pre-existing liver disease [204].

Echinocandins are lipopeptides that inhibit the synthesis of glucan in the cell wall, via noncompetitive inhibition of the enzyme 1,3-β glucan synthase and possess fungicidal activity against *Candida albicans* both *in vitro* and *in vivo* [210]. This antifungal class includes caspofungin, micafungin, and anidulafungin. All have similar spectra of activity and are available only for intravenous administration. Furthermore, each of the echinocandins has an excellent safety profile, as most of the adverse effects involve infusion-related reactions [211]. Regarding the activity against *Candida* biofilm, the echinocandins cited above are able to cause a significant reduction of the *Candida* cell numbers on the implanted catheters using a rat model system and are thus promising for the treatment of biofilm-related infections [212].

5-Fluorocytosine (5-FC) is a fluorinated pyrimidine that has been used to treat candidiasis and other invasive mycoses [213]. Although not used as monotherapy, 5-

FC may be a useful adjunct to amphotericin B or azoles in the treatment of hematogenous candidiasis [214]. Despite the well-known clinical efficacy of 5FC when used in combination with amphotericin B, clinicians are often hesitant to use 5FC due to concerns about toxicity and either primary or secondary resistance [213].

The sordarin (Figure 4.3) family is a class of antifungal agents, characterized by a unique tetracyclic diterpene core including a norbornene system. Unlike traditional antifungal agents, which target only the integrity of the cell membrane through binding of ergosterol or inhibition of its biosynthesis, sordarin acts on elongation factor 2 (EF2). This unusual bioactivity makes sordarin a promising candidate for the development of new fungicidal agent [215]. *In vivo*, therapeutic efficacy of sordarins was seen in nonimmunocompromised murine models of disseminated and mucosal candidiasis although efficacy in a nonimmunocompromised murine model of disseminated aspergillosis was limited [216].

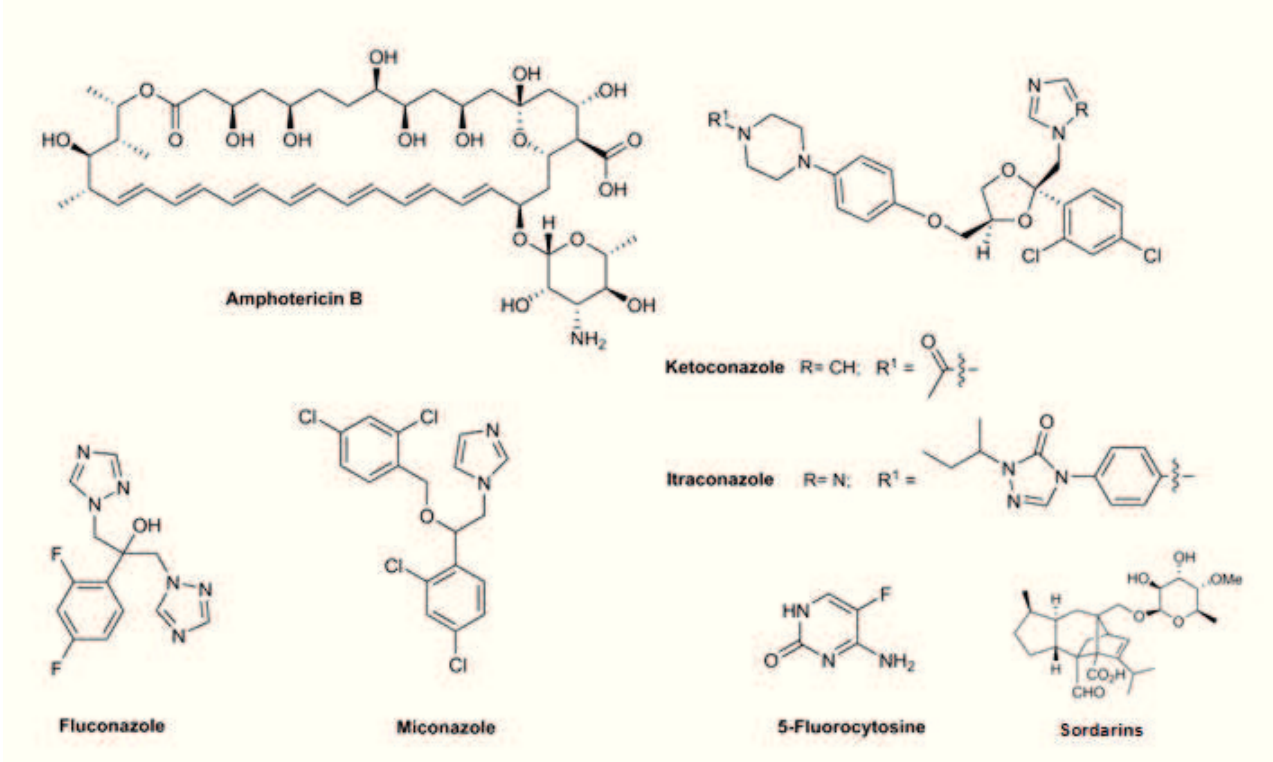

Figure 4.3 Several therapeutic antifungal agents. (Huan Liang. 2008)

4.1.3 AMPs against fungal infection

In recent years, the increased incidence of systemic fungal infection and increased drug resistance to common antifungal agents due to intensive prophylactic and therapeutic uses of these compounds has led to sustained research efforts targeting alternative antifungal compouds. In this regard, antimicrobial peptides and proteins have received increased attention. Lysozyme, lactoferrin, histatins, defensins and cathelicidins are several molecules that have found to be active against yeasts including *Candida albicans* [217].

Lysozyme is an enzyme classically known for its muramidase activity lysing bacterial peptidoglycan and killing bacteria but it is also known for its activity against *Candida albicans*. For example, Wu et al have demonstrated that only high concentrations of lysozyme were able to kill *C. albicans* cells whereas low concentration of this peptide modulate the release of the virulence factor "secreted aspartic protease" (Sap) [218].

Lactoferrin is an iron-binding protein present in various body secretions, including saliva. Human lactoferrin displayed a clear fungicidal effect against *Candida albicans* only under low-ionic strength conditions and this candidacidal activity was inversely correlated with the extracellular concentration of the monovalent cations and was prevented by Na^+ and K^+ (≥30 mM) and by divalent cations Ca^{2+} and Mg^{2+} (≥4 mM). A slight cellular release of K^+, cytosolic acidification, and a change in the membrane potential were observed in *C. albicans* cells treated with lactoferrin, suggesting that this protein directly or indirectly interacts with the cytoplasmic membrane [219]. Moreover, the combination of lactoferrin with fluconazole has been reported to synergistically enhance the antifungal activity of fluconazole against *Candida spp*. and inhibit the hyphal formation in fluconazole-resistant strains of *Candida albicans* [220].

Histatin-1 and -3 are salivary proteins secreted by the parotid and submandibular glands. Histatin-5 is a 24 amino acids long N-terminal fragment of Histatin 3 and is generated by proteolytic cleavage. Histatin-5 has the strongest fungicidal activity, killing yeast and filamentous forms of *Candida species* at 15-30 μM. The mechanism by which Hst 5 causes fungal cell death is still debated. To date, there is a general agreement that Hst 5 must initially interact with and pass through the fungal cell wall before reaching its target to induce toxicity. However, the mode of interaction of Hst-5 with the cell wall and membrane as well as the final target within *Candida cells* have been disputed [221-223]. Histatin-5 (Hst-5) was found to be active against *Candida*

albicans biofilms grown on polymeric surfaces of dentures and other prostheses introduced into the oral cavity. Moreover, Hst-5 pre-coating of acrylic disks caused a significant reduction of biofilm development at 72 h, but not at 24 and 48h, suggesting that Hst-5 acts during the late stages of biofilm development [224].

Human β- defensin 1 (HBD-1), Human β- defensin 2 (HBD-2) and Human β-defensin 3 (HBD-3) were found to be active against *Candida albicans* via the same mechanism of action. Of the three defensins, human β–defensin 3 showed the strongest fungicidal activity against *C. albicans* with a MFC of 2.5 μM and HBD-2 the weakest with a MFC of 8 μM [225]. A membrane permeabilization assay with the nucleic acid stain "Sytox green", which is excluded by intact cells, showed a significant increase in fluorescence after the addition of defensins to *C. albicans*, indicating that at least one mechanism of action of HBD1–3 is increasing the membrane permeability of *C. albicans* [225]. Regarding the activity of defensins in the prevention of *C. albicans* biofilm formation, Pusateri CR. *et al*. have demonstrated that precoating of acrylic disks with hBD-3 did not decrease *C. albicans* biofilm formation at any time point (24, 48, 72 h), despite the 10-fold higher fungicidal activity of HBD-3 as compared to Hst-5 [224].

Next to defensins, the cathelicidins constitute the other larger group of antimicrobial skin peptides. Cathelicidins CRAMP and LL-37 showed fungicidal and fungistatic activity against *C. albicans*, with a similar MIC between 15 and 20 μM [226]. At the skin surface, LL-37 is processed by a serine-protease into shorter peptides such as KS-30 and RK-31. Both peptides showed a higher fungicidal activity against *C. albicans* than LL-37 or CRAMP. A Sytox green permeabilization assay showed that LL-37 and RK-31 render the *Candida* membrane more permeable [226]. The cathelicidin peptides BMAP-27, BMAP-28, SMAP-29, protegrin 1 and indolicidin have shown to posses antifungal activity against more than 70 clinical isolates belonging to over 20 different species of pathogenic fungi some of which were resistant to amphotericin B and azoles. All these cathelicidins rapidly killed *Candida albicans* and *Cryptococcus neoformans* cells in a dose- and time-dependent manner. The rapid uptake of propidium iodide into treated cells and morphological alterations apparent on their cellular surfaces suggest a killing mechanism based on membrane permeabilization and damage [61]. Concerning the activity of cathelicidin peptides against *Candida albicans* biofilm, Tsai PW*. et al.* have demonstrated that LL-37 at sub-inhibitory concentrations was still able to reduce *C. albicans* infectivity by inhibiting *C.*

albicans adhesion to plastic surfaces, oral epidermoid OECM-1 cells, and urinary bladders of female BALB/c mice. They have also shown how this inhibitory effects of LL-37 on cell adhesion and aggregation were mediated by its preferential binding to mannan, the main component of the *C. albicans* cell wall, and partially by its ability to bind chitin or glucan, which underly the mannan layer [227].

4.1.4 AMPs and medical devices

Infections by *Candida spp*, and in particular *Candida albicans*, are increasingly associated with the use of medical devices such as central venous catheters, tracheoesophageal voice prostheses and urinary catheters [228]. On these materials, *Candida albicans* forms biofilms, which are extremely difficult to eradicate using conventional antifungal agents [229]. Several strategies to prevent biofilm formation on polymers have been proposed including surface modification, functionalization with antimicrobial groups and surface coating using antibiotics [230,231]. For urinary catheters, surface coating with silver, gendine (gentian violet and chlorhexidine) and nitrix oxide have been reported [232], but these compounds are cytotoxic and are associated with the development of antibiotic resistance [233]. On the other hand, AMPs could be promising candidates for coating applications due to their broad spectrum antimicrobial activity, good biocompatibility and, above all, the low frequency in selecting resistant strains. Indeed, until now few AMPs including omiganan [234], citropin 1.1 [89] and tachyplesin III [235] have been tested in animal models and in clinical trials for this kind of application with promising results. In this part of my thesis, the activity of two α-helical cathelicidin peptides, i.e. the human LL-37 and its bovine ortholog BMAP-28, against *Candida spp* has been investigated focusing on their anti-biofilm and anti-adhesive propierties.

4.2 MATERIALS AND METHODS:

4.2.1 Peptides

Peptides BMAP-28 (GGLRSLGRKILRAWKKYGPIIVPIIRI-NH2) and LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) were chemically synthesized according to standard methods. The good quality of crude peptides were confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Esquire 4000, Bruker Daltonics Inc., Billerica, MA, USA). Peptide concentrations were determined in aqueous solution by measuring the absorbance at 257 nm for LL-37 and at 280 nm for BMAP-28 taking into account the extinction coefficients of Phe (195 cm^{-1} M^{-1} at 257 nm), and of Trp and Tyr (6839 cm^{-1} M^{-1} at 280 nm).

4.2.2 Other reagents

Miconazole and amphotericin B were purchased from Sigma-Aldrich, resuspended in dimethyl sulfoxide (miconazole) or in water (amphotericin B) and maintained at -20°C until use as reference antifungals.

4.2.3 Silicone disks production

Silicone elastomer (SE) sheets were purchased from G.F. Electromedics (Firenze, Italy). Flat circular disks, 0.5 cm in diameter, were obtained by cutting with a cork borer, rinsed extensively with distilled water and autoclaved following the manufacturer's instructions. Sterilized disks were transferred to 96-well plates immediately before use.

4.2.4 Fungal isolates and culture conditions

Fungal organisms used in this study included the reference strain *Candida albicans* SC5314 and 27 clinical isolates, including 12 *Candida albicans*, 7 *Candida glabrata*, 3 *Candida krusei*, one *Candida norvegensis*, one *Candida parapsilosis* and 3 *Saccharomices cerevisiae*. *Candida* and *Saccharomices* isolates were cultivated from vaginal swabs collected from patients with vaginitis at the Microbiology Unit of the University Hospital of Udine. The strains were characterized using EUCAST test and tested against amphotericin B, fluconazole, itraconazole, ketoconazole, 5-fluorocytosine and voriconazole using a broth microdilution assay in accordance with the NCCLS M27A protocol . Fungal cells were cultured on Sabouraud dextrose agar

plates (Oxoid, UK) at 30°C for 24-48 h, maintained at 4°C and subcultured on Sabouraud dextrose agar plates prior to use.

4.2.5 Planktonic antifungal susceptibility testing

Yeast cells were resuspended in Sabouraud liquid medium or in vaginal simulated fluid (VSF) adjusted to pH 4.2, 5.5 or 6.5. The composition (g/l) of VSF was as follows: NaCl, 3.51; KOH, 1.40; $Ca(OH)_2$, 0.222; bovine serum albumin, 0.018; lactic acid, 2.00; acetic acid, 1.00; glycerol, 0.16; urea, 0.4; glucose, 5.0 [236]. Cell density was assessed by measuring turbidity at 600 nm and was adjusted to obtain the proper inoculum size. Minimum inhibitory concentrations (MICs) were determined using the broth microdilution method [237]. Briefly, two-fold serial dilutions of each test agent, either in Sabouraud liquid medium or in VSF, were prepared in 96-well microtiter plates to a final volume of 50 µl. A total of 50 µl of the adjusted inoculum was added to each well to achieve the final concentration of $5x10^4$ cells/ml (or $1x10^7$ cells/ml, when MICs against biofilm-forming concentrations of *C. albicans* SC5314 were to be determined). Samples were then incubated at 30°C for 48 h. The MIC was taken as the the lowest concentration of the test agent that resulted in complete inhibition of visible growth after 48 h incubation. Minimum fungicidal concentration (MFC) were determined by transferring 50 µl aliquots of each sample treated with concentrations higher than the MIC into Sabouraud dextrose agar plates, and incubating plates at 30°C for 24 h.

4.2.6 Assessment of biofilm formation

Candida biofilms were formed as described by [186] with some modifications. Briefly, overnight cultures of *C. albicans* SC5314 were adjusted with Sabouraud-dextrose broth to give a 1×10^7 CFU/ml suspension and then incubated at 100 µl/well into flat bottom 96 well polystyrene microtiter plates (Sarstedt), or into SE disk-containing 96 well microtiter plates, at 37°C for 48 h. At the end of the incubation time non-adherent cells were removed and wells were gently rinsed with PBS. Biofilms were quantified using the XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] reduction assay as a direct measure of the metabolic activity of adherent cells. The XTT assay was adapted from . Briefly, XTT was prepared at 2 g/liter in PBS and filter sterilized through a 0.22-µm-pore-size filter. Menadione (Sigma-Aldrich) was added to 20 µM final concentration. A 100-µl aliquot

of XTT-menadione solution was added to each biofilm-containing well. Microtiter plates were incubated in the dark for 30 min at 37°C. Changes in the absorbance of XTT were measured spectrophotometrically at 490 nm using a microtiter plate reader (BioTek Instruments Inc.). The percentage of viable cells was calculated as: (OD_{490} of treated cells/OD_{490} of untreated cells) × 100.

4.2.7 Biofilm formation inhibition assay

Planktonic *C. albicans* SC5314 cells at 1×10^7 CFU/ml in Sabouraud liquid medium were dispensed at 100 µl/well into 96-well polystyrene microtiter plates or in SE disk-containing 96-well polystyrene microtiter plates, and incubated at 37°C for 30 min or 48 h in the absence and presence of each test agent at MIC and half-MIC dose for 1×10^7 CFU/ml *C. albicans* SC5314. Alternatively, SE disks were pre-coated with peptides by incubating SE disk-containing wells for 1 h at 37°C with 100 µl Sabouraud liquid medium in the absence and presence of 8 µM, 16 µM and 32 µM BMAP-28 and of 16 µM, 32 µM and 64 µM LL-37. Unbound peptide was removed from the wells by aspiration after 1 h incubation, wells were carefully rinsed with PBS, *C. albicans* SC5314 cells were dispensed onto peptide-coated SE disks at 1×10^7 CFU/ml in peptide-free Sabouraud liquid medium, and incubated at 37°C for 48 h. At the end of the incubation times, adherent cells were quantified by XTT after removing non-adherent cells by aspiration and rinsing with PBS. Total (i.e., adherent and non adherent) cell numbers were determined in replica samples run in parallel, following microtiter plate centrifugation at 1300×g to sediment non-adherent cells.

4.2.8 Antifungal susceptibility testing of *Candida* biofilms

Mature (i.e., 48-h-old) *C.albicans* SC5314 biofilms were incubated for 48 h at 37°C in Sabouraud liquid medium, in the absence and presence of doubling concentrations of BMAP-28, LL-37, MCZ and AMB. At the end of the incubation period, non-adherent cells were removed by aspiration, wells were carefully rinsed with PBS and the metabolic activity of adherent cells was quantified using the XTT assay. Decrease in biofilm cell viability was assessed as a decrease in absorbance at 490 nm, compared with untreated biofilms. Sessile minimum inhibitory concentrations (SMICs) were determined at 50% and 90% biofilm cell inhibition. For time-kill studies, *C.albicans* SC5314 biofilms were exposed to 16 and 32 µM BMAP-28 and 16 and 32 µM MCZ at 37°C, for 15 and 90 min and for 3, 6, 24 and 48 h. At the end of the

incubation times, samples were processed as above for quantification of metabolic activity of adherent cells by XTT.

4.2.9 Fluorescence Microscopy

Mature (48-h-old) *C. albicans* SC5314 biofilms in 96-well microtiter plates were incubated for 90 min with BMAP-28 or MCZ at 16 and 32 μM in Sabouraud medium. The medium was then replaced with fresh medium containing 1 μg/ml propidium iodide and 25 μg/ml concanavalin A Alexa Fluor® 488 conjugate (Life Technologies) and plates were incubated for 40 min at 37°C. Stained biofilms were examined under an inverted Leica DMI6000RB epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) placed in a climate chamber at 37°C and images were captured with a Micromax charge-coupled device camera (Roper Scientific).

4.2.10 Statistical Analysis

Statistical differences among groups of data were analysed by one-way analysis of variance followed by the Bonferroni post test, using GRAPHPAD Prism version 5.0. In all comparisons, $P<0.05$ was considered significant.

4.3 RESULTS:

4.3.1 Antifungal activity against clinical isolates of *S. cerevisiae* and *Candida spp*

4.3.1.1 Antifungal activity in standard conditions

The antifungal activity of the α-helical cathelicidins BMAP-28 and LL-37 was tested *in vitro* against wild-type *C. albicans* SC5314 as a reference strain and against 27 yeast strains obtained from vaginal exudates of patients with vaginal yeast infection. The isolates included 3 strains of *Saccharomyces cerevisiae* and 24 *Candida* strains half of which were non-albicans strains (Table 4.1). All S. cerevisiae and *C.albicans* strains were azole-susceptible. Apart from one azole-susceptible *C. parapsilosis*, the non-albicans *Candida* isolates were resistant (R) or susceptible dose-dependent (SDD) to azoles, and were identified to the species level as follows: *C. glabrata* (6/7 SDD and 1/7 R to ITC; 2/7 SDD to FLC), *C. krusei* (3/3 SDD to ITC; 2/3 SDD and 1/3 R to FLC), *C. norvegensis* (1 isolate SDD to ITC and to FLC).

BMAP-28 and LL-37 revealed comparable potencies against *S. cerevisiae* (Table 4.1). However their activities against *Candida* strains varied considerably. BMAP-28 was effective in the low micromolar concentration range against *C. albicans* SC5314 and against all *Candida* isolates but *C. glabrata* CG-2, CG-5, CG-6 and CG-7 (Table 4.1). LL-37 did not inhibit the growth of *C. albicans* SC5314 at up to 64 μM and was inactive or poorly active against most *Candida* isolates (Table 4.1).

Table 4.1. Antifungal activity of BMAP-28 and LL-37 against vaginal isolates of *Candida spp* and *S. cerevisiae*.
[a]MIC value at which ≥50% of *Candida* isolates are inhibited
[b]MIC value at which ≥90% of *Candida* isolates are inhibited

	MIC (μM)	
Organism	BMAP-28	LL-37
C. albicans SC5314	2	> 64
C. albicans CA-1	2	> 64
C. albicans CA-2	2	16
C. albicans CA-3	4	> 64
C. albicans CA-4	8	> 64
C. albicans CA-5	2	16
C. albicans CA-6	2	> 64
C. albicans CA-7	2	16
C. albicans CA-8	2	64
C. albicans CA-9	2	> 64
C. albicans CA-10	4	> 64
C. albicans CA-11	2	32
C. albicans CA-12	4	> 64
MIC_{50}[a]	2	> 64
MIC_{90}[b]	4	> 64
C. glabrata CG-1	8	> 64
C. glabrata CG-2	32	> 64
C. glabrata CG-3	8	> 64
C. glabrata CG-4	4	> 64
C. glabrata CG-5	32	> 64
C. glabrata CG-6	32	> 64
C. glabrata CG-7	> 64	> 64
MIC_{50}[a]	32	> 64
MIC_{90}[b]	32	> 64
C. krusei CK-1	2	4
C. krusei CK-2	4	32
C. krusei CK-3	8	64
C. norvegensis	8	16
C. parapsilosis	2	32
S. cerevisiae SC-1	2	2
S. cerevisiae SC-2	2	2
S. cerevisiae SC-3	2	2

4.3.1.2 Antifungal activity in Vaginal Simulated Fluid (VSF)

The antifungal effects of BMAP-28 and LL-37 on representative isolates of *C. albicans* and *C. krusei* were also investigated in synthetic vaginal simulated fluid (VSF) [236] at pH values ranging from 4.2 to 6.5 to mimic the physical and chemical properties of vaginal secretions. *C. glabrata*, *C. parapsilosis* and *C. norvegensis* were not tested in VSF because of poor cell growth in this medium. As shown in Table 4.2, the antifungal effects of BMAP-28 against *C. albicans* and *C. krusei* were substantially preserved in VSF although, lowering the pH from 6.5 to 4.2 resulted in 2- to 3-fold increase in MIC values. LL-37 was ineffective up to 64 μM against all *Candida* strains tested (not shown).

The activity of BMAP-28 against susceptible *Candida* isolates was further examined in terms of minimum fungicidal concentration (MFC), to ascertain whether the antifungal susceptibility testing correlated with killing capacity. For all *Candida* isolates, the MFC values of BMAP-28 were equal to MIC values, consistent with powerful candidicidal activity.

Table 4.2 Anti-*Candida* activity of BMAP-28 in vaginal simulated fluid.

	MIC (μM)			
	Sabouraud	VSF		
Organism	pH 5.5	pH 6.5	pH 5.5	pH 4.2
C. albicans SC5314	2	8	16	32
C. albicans CA-5	4	8	16	32
C. albicans CA-6	2	16	16	32
C. krusei CK-1	2	16	32	32
C. krusei CK-2	4	16	16	32

4.3.2 Activity against pre-formed *Candida* biofilm

4.3.2.1 Evaluation of Sessile MICs (SMICs) of the peptides and antifungal drugs

Evidence from a mouse model of vaginal infection indicates that *Candida* cells have the ability to adhere to vaginal epithelium and produce biofilm. Biofilm are structured microbial communities characterized by a network of adherent cells connected by water channels and encapsulated within an extracellular matrix, that renders the cells less susceptible to antimicrobial agents ,

Since this mode of growth in *Candida* confers increased resistance to antifungal agents we were interested in examining the activity of BMAP-28 and LL-37 against sessile *Candida* cells relative to that of conventional anti *Candida* agents such as miconazole (MCZ) and amphotericin B (AMB). The anti-biofilm activity was quantified using the XTT reduction assay. As reported in Table 4.3, BMAP-28 effectively reduced the viability of mature *C. albicans* SC5314 biofilms grown on the surface of microtiter plates, with $SMIC_{50}$ and $SMIC_{90}$ of 8 μM and 16 μM following 48 h biofilm treatment. MCZ was somewhat less efficient than BMAP-28 showing a $SMIC_{50}$ and $SMIC_{90}$ of 16 μM and 32 μM, and LL-37 and AMB were ineffective at concentrations up to 32 μM (Table 4.3).

Table 4.3. Activity of BMAP-28, LL-37, MCZ and AMB against *C. albicans* SC5314 biofilm

Antifungal agent	$SMIC_{50}$ * (μM)	$SMIC_{90}$ * (μM)
BMAP-28	8	16
LL-37	> 32	> 32
MCZ	16	32
AMB	> 32	> 32

*$SMIC_{50}$ and $SMIC_{90}$ were defined as the lowest concentration of test compound resulting in at least 50% or 90% reduction in metabolic activity after 48h treatment, compared with the untreated control.

4.3.2.2 Time Kill Kinetics of BMAP-28 and MCZ

Time-kill studies were used to better understand the mechanism of action of the two active compound (BMAP-28 and MCZ) against preformed biofilm. BMAP-28 at 16 and 32 μM induce an initial rapid drop in biofilm cell viability, with approximately 55% and 25% live cells after 90 min treatment, and virtually complete killing after 48 h treatment (Figure 4.1). Compared with BMAP-28, MCZ at 16 and 32 μM exhibited a significantly slower killing kinetics although, most biofilm cells were inactivated after 48 h (Figure 4.1).

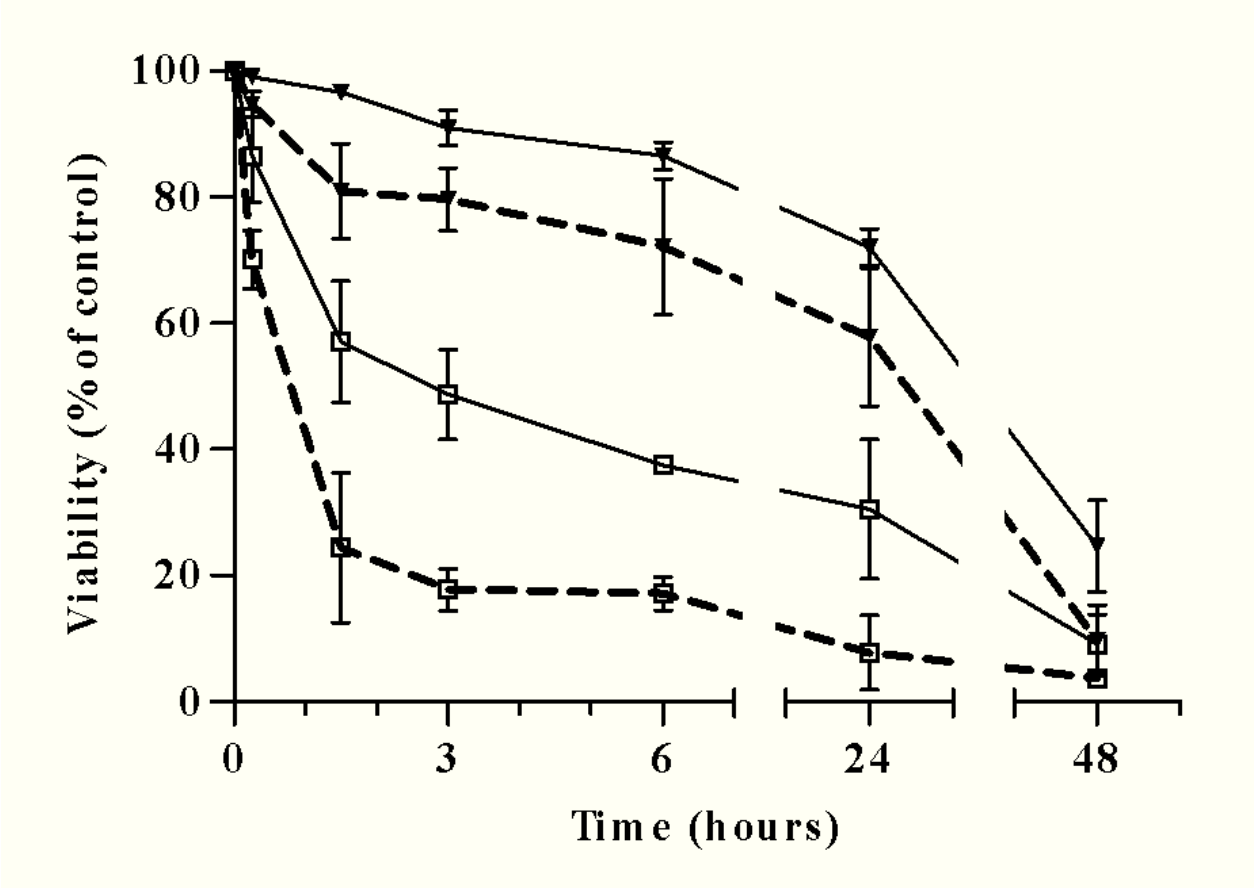

Figure 4.1 Killing kinetics of BMAP-28 and MCZ against *C. albicans* SC5314 biofilm.
C. albicans SC5314 biofilms were incubated in the presence of BMAP-28 (open squares) or MCZ (close triangles) at 16 μM (continuos line) and 32 μM (dashed line), for the indicated time periods. At the end of each incubation period, biofilm cell viability was assessed using the XTT assay. Results are reported as percent viable cells relative to untreated samples and are the mean ± SD of three independent experiments performed in triplicate.

4.3.2.3 Morphology of the treated biofilm

The ability of BMAP-28 to rapidly kill *Candida* cells within the biofilm structure was further assessed by fluorescent microscopy examination of 48-h-old *C. albicans* SC5314 biofilms stained with PI to reveal cell membrane damage, following 90 min incubation in the absence and presence of BMAP-28 or MCZ at 16 and 32 μM. A representative image of biofilm exposed to BMAP-28 is shown in Figure 4.2 panel B. It shows a clearly detectable red fluorescence suggesting disseminated cell permeabilization. In contrast, virtually no PI-positive cells were detected in untreated (Figure 4.2 A) and in biofilms treated with MCZ, AMB and LL-37 (not shown).

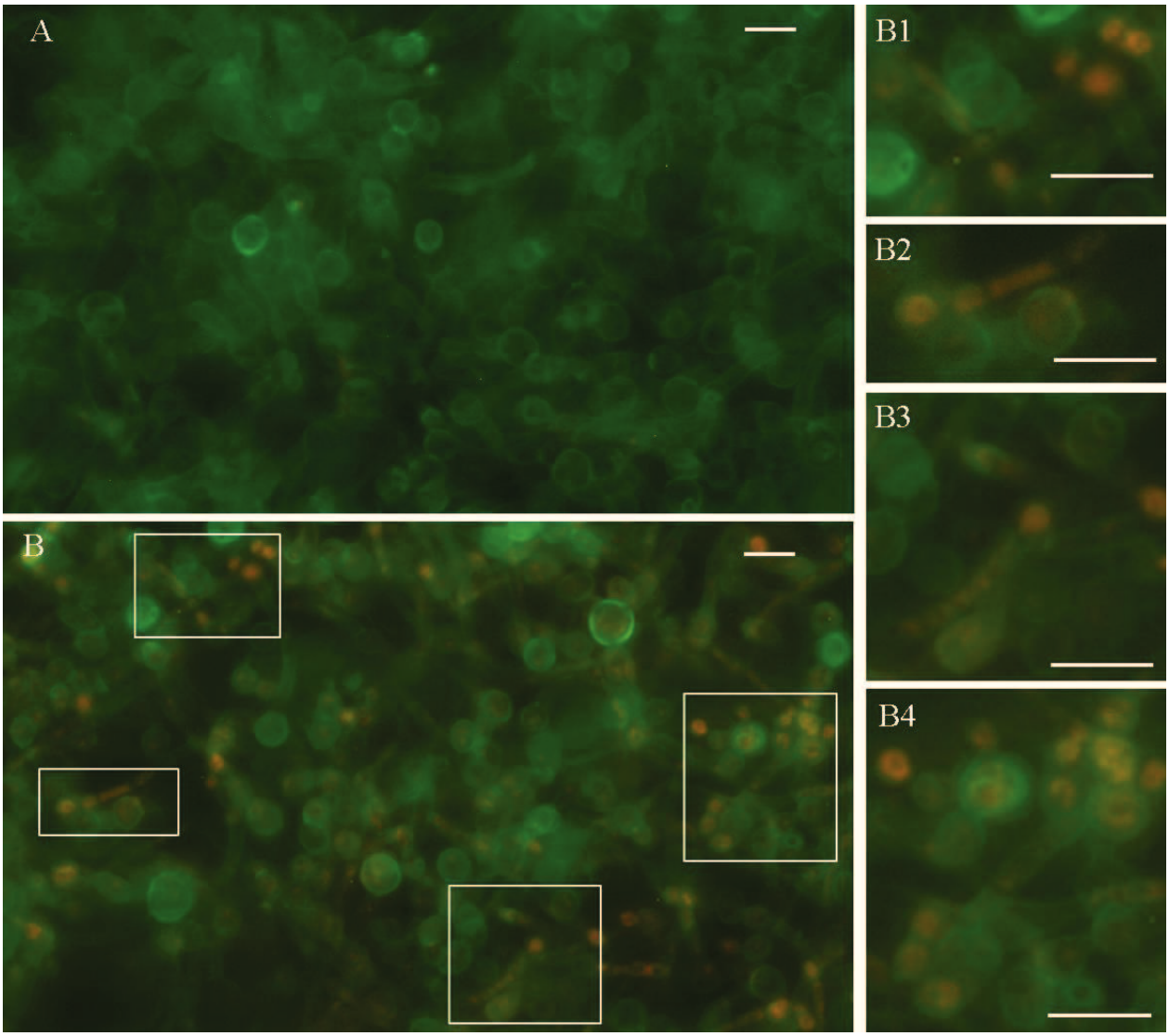

Figure 4.2 Fluorescence microscopy images of *C. albicans* biofilms.
Mature, 48-h-old C. albicans SC5314 biofilms were incubated for 90 min at 37°C in the absence (A) and presence (B) of 16 μM BMAP-28, and then stained with propidium iodide and concanavalin A Alexa Fluor® 488 conjugate. Magnification, x40. Boxed regions in (B) are presented at x2 magnification in B1-B4. Bars, 10 μm. FM was performed three times on two coverslips per condition.

4.3.2.4 *Effect of BMAP-28 against biofilm produced by* Candida *clinical isolates*

To confirm and extend our investigation of the anti-*Candida*-biofilm activity of BMAP-28, representative clinical vaginal isolates of *C. albicans*, *C. krusei* and *C. glabrata* were grown in sessile mode for 48 h and treated for 24 h with 16 μM BMAP-28. The killing activity of the peptide against *C. albicans* and *C. krusei* biofilms was comparable to or higher than against *C. albicans* SC5314 (Figure 4.3). However the peptide at 16 μM was virtually ineffecive against *C. glabrata* CG-4 biofilm (Figure 4.3) although this isolate was susceptible to BMAP-28 under planktonic growth conditions.

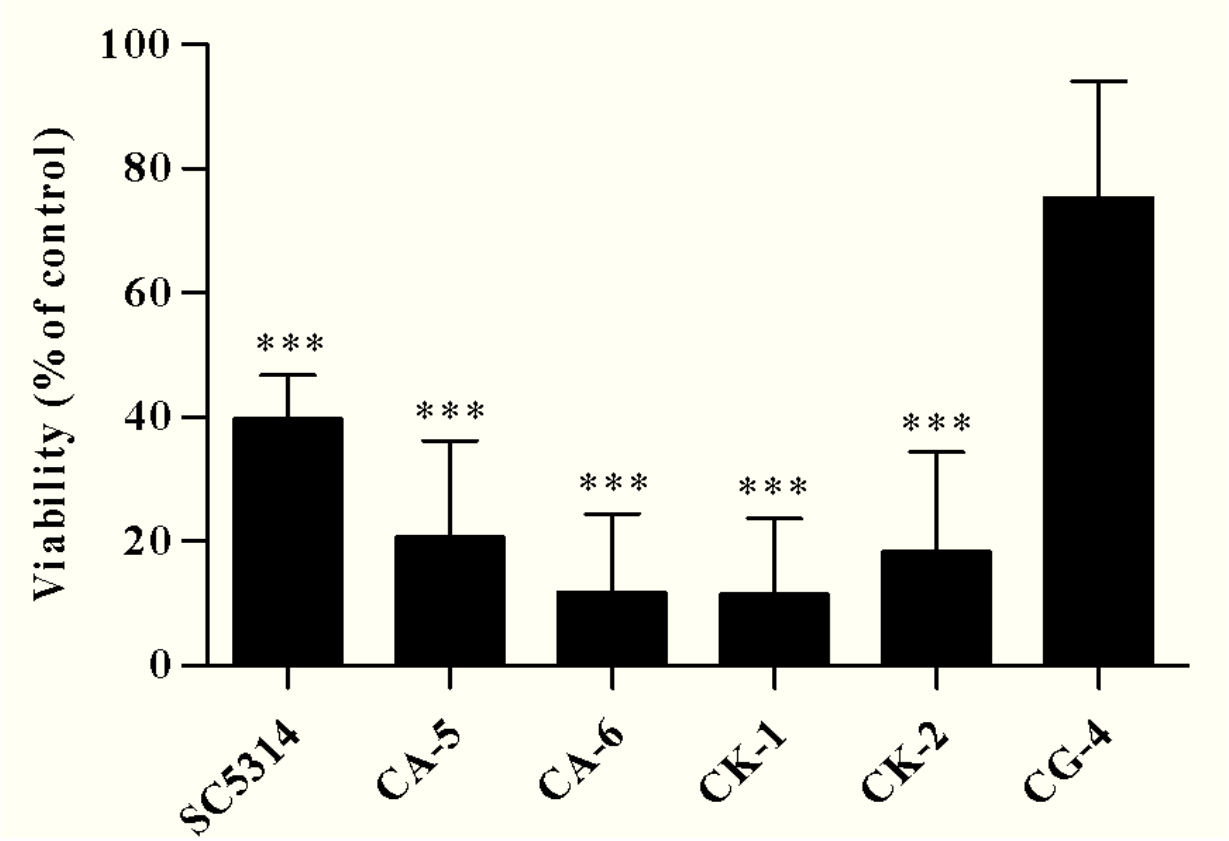

Figure 4.3 Effect of BMAP-28 against biofilms formed by vaginal *Candida* isolates.
Biofilms formed by *C. albicans* SC5314 and by clinical isolates of *C. albicans* (CA), *C. krusei* (CK) and *C. glabrata* (CG) were incubated at 37 °C in the presence of 16 μM BMAP-28 for 24 h. Cell viability was assessed using the XTT assay. Results are reported as percent viable cells relative to untreated samples and are the mean ± SD of at least three independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

4.3.3 Effect on biofilm formation

Having examined the activity against preformed biofilms, we asked whether the compounds under study have the capacity to block the initial step in the formation of *Candida* biofilm so as to prevent biofilm production. We also asked which was the activity of these compounds in a material different to polystyrene. For this reason we have also used medical grade silicone, that is an important material due to its widespread application in medical devices.

4.3.3.1 Polystyrene surfaces

Planktonic *C. albicans* SC5314 cells were seeded into microtiter plates at $1x10^7$ CFU/ml for biofilm formation and each test compound was added to the culture medium immediately afterwards, at concentrations corresponding to the MIC exhibited against $1x10^7$ CFU/ml *C. albicans* SC5314 cells (i.e., ≥ 16 μM, 64 μM, 32 μM and 2 μM for BMAP-28, LL-37, MCZ and AMB), and one-half MIC. Plates were incubated at 37° C for 30 min or 48 h, and adherent vs total viable cell numbers were determined at the end of each incubation period using the XTT assay.

As can be observed in Figure 4.4 A, both BMAP-28 and LL-37 produced a marked decrease in the number of adherent viable cells compared with untreated control samples, already at the early time point. BMAP-28 also caused a significant decrease in total (i.e., adherent and non-adherent) cell numbers (Figure 4.4 C), suggesting it prevented establishment of *Candida* biofilms by rapidly killing planktonic cells. At the 30 min time point, LL-37 at MIC caused an approximately 80% reduction in adherent cell numbers (Figure 4.4 A). However unlike BMAP-28, LL-37 hardly affected total cell numbers even at the late time point (Figure 4.4 D). These results suggest that LL-37 acted primarily by inhibiting cell adhesion to the plastic surface rather than by killing planktonic cells. AMB was highly effective in inhibiting biofilm formation at the late time point by affecting total cell numbers (Figure 4.4 B and D). In contrast, MCZ only exhibited modest inhibitory effects.

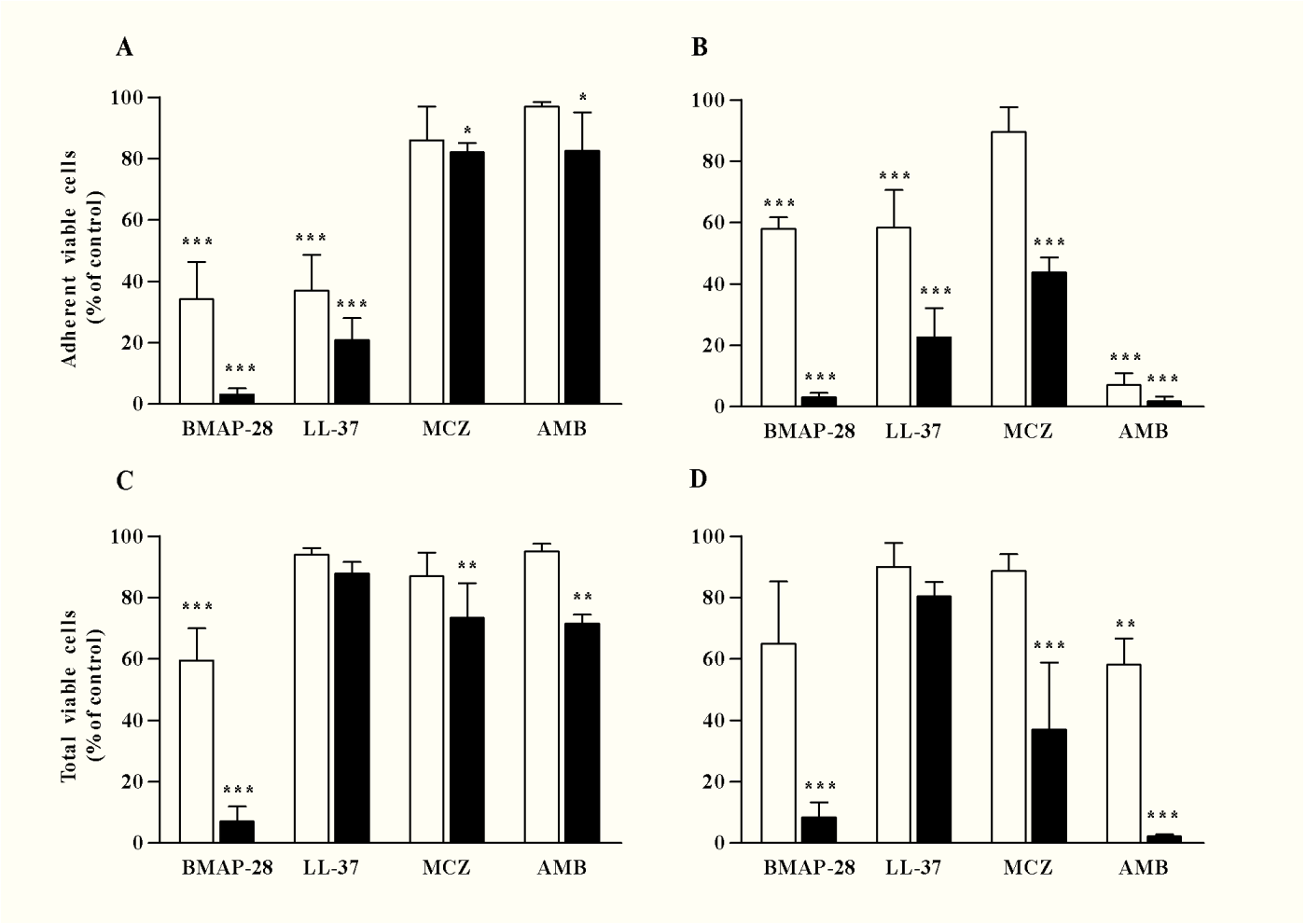

Figure 4.4 Inhibition of *C. albicans* biofilm formation on polystyrene plates.
C. albicans SC5314 cells were seeded at $1x10^7$ cells /ml into 96-well polystirene microtiter plates and incubated for 30 min (A, C) or 48 h (B, D) in the absence and presence of LL-37, BMAP-28, AMB or MCZ at concentrations corresponding to ½ MIC (open bars) and MIC (solid bars) against $1x10^7$ planktonic cells /ml. Adherent (A, B) and total (C, D) *Candida* cell numbers were quantified by the XTT assay. Results are reported as the mean ± SD of at least three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.3.3.2 Medical grade silicone surfaces

BMAP-28 and LL-37 were further tested for ability to prevent *C. albicans* SC5314 biofilm formation on the surface of medical grade silicone elastomer (SE) disks - a material which has found widespread use in medical device manufacturing industries - placed into microtiter wells. Peptides were added to the cell culture medium immediately after seeding cells onto uncoated SE disks (Figure 4.5 A) or, alternatively, cells were dispensed on peptide-coated SE disks and incubated in

peptide-free Sabouraud medium (Figure 4.5 B). Cell viability was determined following 48 h cell incubation at 37° C.

When added to the medium immediately after cell seeding, BMAP-28 at 16 µM effectively reduced the number of adherent and total *Candida* cells, whereas LL-37 up to 64 µM only showed anti-adhesive activity (Figure 4.5 A). A dose-dependent decrease in the number of viable adherent cells was detected when cells were seeded on BMAP-28 or LL-37 coated disk. Under this condition, neither peptide affected the viability of total *Candida* cells (Figure 4.5 B).

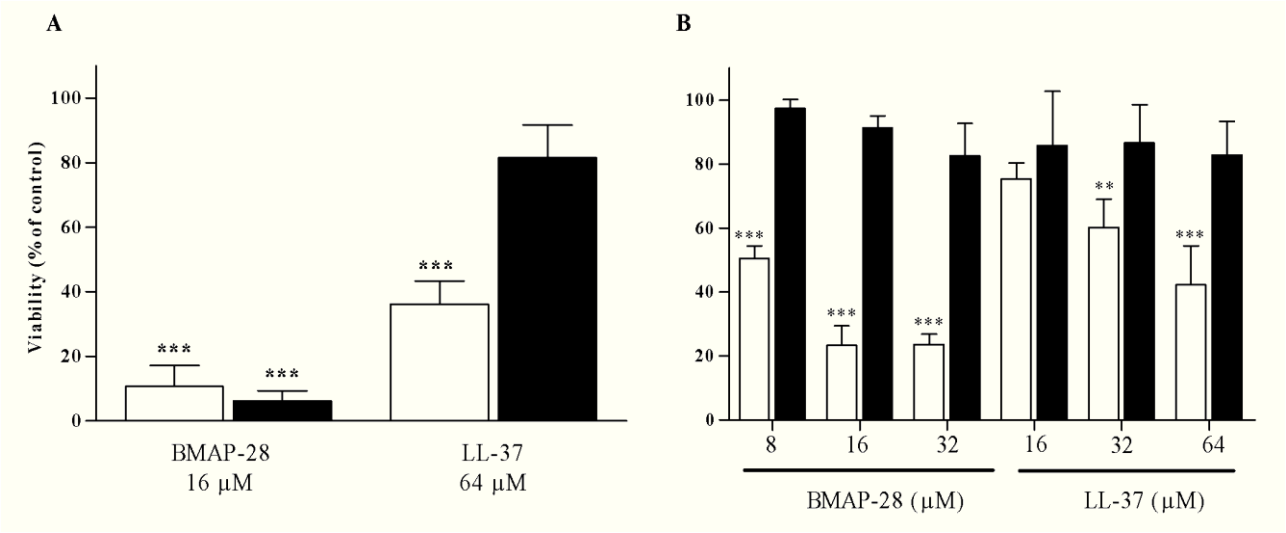

Figure 4.5 Inhibition of *C. albicans* biofilm formation on silicone surface.
C. albicans SC5314 cells were seeded onto SE disks and incubated in the absence and presence in the culture medium of 16 µM BMAP-28 or 64 µM LL-37 (A), or were deposited onto SE-disks preincubated with the indicated peptide concentrations and then rinsed to remove unbound peptide prior to cell seeding (B). Following incubation at 37°C for 48 h, the number of adherent (open bars) and total (solid bars) cells was quantified by the XTT assay. The results are reported as the mean ± SD of at least three independent experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

4.4 DISCUSSION:

Candida spp are the most common cause of fungal infection leading to mucocutaneous diseases. Vulvovaginal candidiasis affects approximately 75% of fertile age women with 5-10% incidence of recurrent infection [126]. Importantly, planktonic *Candida* cells tend to adhere to both biological and non biological surfaces, and their ability to form stable biofilms often results in serious medical device-related infections [228]. Given the growing threat of drug resistance to the most common treatments, there is a great demand for novel anti *Candida* agents. Cathelicidins are antimicrobial peptide components of the innate immune system of mammals. Broad-spectrum antimicrobial activity and low propensity to select resistant strains make these compounds interesting candidates for developing novel antifungals [238].

In the present work, the human cathelicidin peptide LL-37 and its bovine orthologue BMAP-28 were tested to determine their *in vitro* activity against planktonic and biofilm cells of *Candida spp*. The activity of these peptides was compared to that of conventional anti *Candida* drugs such as fluconazole, itraconazole, miconazole and amphotericin B.

We show that BMAP-28 exhibited good fungicidal activity against most planktonic *Candida* isolates and retained substantial activity in vaginal simulated fluid. These results are consistent with previous studies indicating ability of BMAP-28 to inactivate *in vitro* a variety of pathogenic fungi grown in planktonic form [61,124], also including a collection of *Candida* species isolated from clinical samples. In this respect, we show that BMAP-28 is effective against various azole-resistant non-albicans *Candida* isolates. Our finding possibly reflects the distinctive nature of the interaction of this peptide with the yeast membranes. Unlike antifungal azoles, that act by interfering with the biosynthesis of membrane ergosterol, amphipathic AMPs such as BMAP-28 exert their antimicrobial effects via membrane binding (insertion) and permeabilization [21]. The latter mechanism is faster and somewhat less specific than the azole mechanism and thus, the development of microbial resistance to AMPs is generally less likely to arise.

In spite of its structural and evolutionary relatedness to BMAP-28, LL-37 was virtually ineffective against most BMAP-28-sensitive *Candida* strains. Indeed, a relevant number of studies has demonstrated that individual differences in size and sequence among AMPs sharing a common structural scaffold can significantly affect

the antimicrobial specificities [24,168]. In the case of BMAP-28 and LL-37, these differences have resulted in distinct structuring and aggregational properties. BMAP-28 behaves as a random-coil monomer in physiological solution and only adopts a helical conformation when interacting with membranes while conversely, LL-37 has a salt-dependent ability to structure and aggregate in saline solution [239]. This distinct behaviour influences the mode of membrane interaction and membrane damage of the two peptides. Specifically, the aggregate-forming tendency of LL-37 would favour non-specific binding to medium components and microbial surfaces, with negative effects on its lytic capacity, while the propensity of BMAP-28 to structure at the membrane level would allow for efficient penetration across cell-wall components.

In our study we observed that BMAP-28 was active against *C. albicans* isolates, (MIC_{50}, 2 μM) but to a minor extent to *C. glabrata* isolates (MIC_{50}, 32 μM). We speculate that the reduced antifungal activity of BMAP-28 against *C. glabrata* could be probably a consequence of the thicker cell wall of *C. glabrata* with respect to *C. albicans* [240], also related to the low activity of this peptide against *C. glabrata* preformed biofilm (about 30% of killing activity).

Jang WS. *et al.* have demonstrated that Histatin 5, an antimicrobial peptide endowed with a strong fungicidal activity against *Candida spp*, requires the initial binding to exposed β-1-3-glucans in the cell to exert activity against *Candida albicans* cells [241]. Moreover, Tati S. *et al.* have observed that β-1-3-glucan are also important in Histatin-5 interactions with *C. glabrata* [242].

As Biofilms are notoriously difficult to eliminate and they are a source of many recurrent infections also due to reduced susceptibility to the common antifungal agents [179,196,208,243,244] we were interested in examining the antimicrobial activity of BMAP-28 and LL-37 compared with two common antifungal agents (Miconazole and Amphotericin B) against biofilm formed by *C. albicans* SC5314, a *C. albicans* strain frequently used as a wild-type control [197,202,227,243,245]. In our *in vitro* studies BMAP-28 and MCZ ($sMIC_{50}$ 8 μM and 16 μM), but not LL-37 and AMB ($sMIC_{50}$ >32 μM), effectively reduced mature biofilm viability although, BMAP-28 was faster-acting and more potent than MCZ as shown in time killing studies. In line with the results obtained by Khan MSA and Ahmad. I. 2012 [202], the sMIC of AMB was increased about 256-fold over the planktonic MIC in *C. albicans* SC5314 (0.25 μM). The low activity of AMB against preformed biofilm could be explained by the decreased levels of ergosterol in sessile *C. albicans* [208], being the ergosterol the primary membrane

target for this antifungal agent [246]. Changes in sterol profile may lead to altered membrane permeability and hence prevent or retard the entry of this antifungal agent into candidal cells [208].

Data obtained by fluorescence microscopy have shown that BMAP-28 was active against preformed *C. albicans* biofilm by a membranolytic mechanism of action. To the other hand, miconazole, a drug that inhibits the growth of *Candida* by negatively affecting the ergosterol biosynthesis [246], did not cause membrane permeabilization, suggesting a mode of action distinct from that of BMAP-28. In this regard Vandebosch D. *et al*. have recently observed that the production of reactive oxygen species was strongly increased in sessile *Candida* cells treated with 5mM miconazole, indicating that ROS may be also responsible for the fungicidal effect of this antimycotic agent [247]. The activity of BMAP-28 against preformed *C. albicans* biofilm is comparable with that of the synthetic antimicrobial peptide KSL-W, although they possess different mechanisms of action [248]. Importantly, BMAP-28 at its $sMIC_{90}$ efficiently killed not only the biofilms formed by the reference strain *C. albicans* SC5314, but also the sessile form of most biofilm-forming *Candida* isolates.

In addition to the results obtained against preformed biofilms, our findings indicate that BMAP-28 reduced biofilm formation to both polystyrene and medical grade silicone surfaces primarily by killing planktonic cells, whereas LL-37 inhibited only cell adhesion without affecting total cells. The latter finding seems in line with Chang HT, *et al*, wich have shown that LL-37 decrease cell adhesion by interacting with the cell-wall β-1,3-exoglucanase Xog1p, which is involved in cell-wall β-glucan metabolism [249].

To date, the use of AMPs as coating materials on the surfaces of medical devices to prevent biofilm formation have been pointed out by several studies and has received increased attention [224,250,251]. In this regard we have studied the capacity of BMAP-28 and LL-37 to inhibit biofilm formation when biofilm was grown on medical grade silicone surfaces pre-coated with these AMPs. We observed that both peptides were able to produce a decrease in the number of adherent cell at 48h suggesting that they probably function as pellicle component that influences adhesion of *C. albicans*.

To my knowledge, this is the first time that the cathelicidin peptide BMAP-28 was studied for its antimicrobial activity against *Candida* biofilms. We have observed that BMAP-28 was highly effective against all *Candida* isolates also including azole-

resistant strains, produced a fast and marked reduction of the growth of mature biofilm and inhibited biofilm formation on abiotic surfaces, whereas LL-37 prevented biofilm formation by inhibiting cell adhesion on abiotic surfaces. In conclusion, the use of BMAP-28 and LL-37 as coating material on silicone medical devices (e.g. indwelling urinary catheters) could be a promising therapeutical approach and is worth further investigation towards this aim.

5 PREVENTION OF FISH INFECTIONS IN AQUACULTURE

5.1 INTRODUCTION:

5.1.1 Aquaculture and related problems

With the decreasing numbers of wild fisheries [252] there is an increasing demand for seafood that could be filled by commercial aquaculture. Aquaculture is now a major global industry with total annual production exceeding 50 million tonnes and estimated value of almost US$ 80 billion [253] With an average annual growth of 6-9% from 1970–2007, it has been the fastest growing animal food-producing sector [253]. As a consequence of this increased demand of seafood, the aquaculture practices are currently intensified. This means that fish must be grown in a relatively small space and as fast as possible, leading to a modification of normal conditions (e.g., dissolved oxygen, ammonia, nitrite, fish density and nutritional status) which are no longer suitable for the physiological functioning of the fish. Beyond a certain level a stressed state occurs, leading to a decrease in immune capabilities that render fish more susceptible to pathogens [254]. In fact, in recent years a rapid and uncontrolled growth of aquatic pathogens with subsequent indiscriminate use of antibiotics has been observed in fish farms. Presently, these two factors are the most important concerns for both researchers and farmers. Diseases caused by infectious micro-organisms are known to be one of the major constraints in the aquaculture industry for past many years and are impeding the development and sustainability of the industry throughout the world [255]. For example, Frans I *et al* [256] have shown that the gram-negative bacterium *Vibrio anguillarum* causes vibriosis in both fresh and salt-water fish species including various species of economic importance to the larviculture and aquaculture industry, such as salmon, *Salmo salar*; rainbow trout, *Oncorhynchus mykiss* (Walbaum); turbot*, Psetta maxima*(L.); sea bass, sea bream, *Sparus aurata*. Aside from *Vibrio anguillarum*, other pathogens have been found of relevance in aquaculture including Nodavirus, Yersinia ruckeri and Aeromonas hydrophila [257-

259]. To summarize, all these problems have prompted the researchers to explore new and alternative avenues to replace antibiotic use against pathogens in order to find suitable tools to control the diseases in this sector. Current approaches in this regard include vaccines, dietary supplement with probiotics, prebiotics, and immunostimulants to boost fish immune defences

According to aquaculture production statistics published by FEAP (2013), Italy is the sixth largest fish producer in the European Union having a total quantity for 2012 estimated around 60,000 tons. This FEAP production report include data on the following species reared in European aquaculture: atlantic salmon, rainbow trout, halibut and cod, catfish, sturgeon and eel, see bass, see bream, turbot and sole. Among these species, rainbow trout is the major farmed fish species in Italy and the second one in Europe. Indeed, the rainbow trout represented about 65% of the Italian farmed fish in the years 2003-2012 [260]. In Italy there are about 300 operational freshwater fish farms that are concentrated in the north of the country (Veneto, Friuli Venezia Giulia, Trentino Alto Adige, Lombardy, Emilia-Romagna and Piemonte regions) and the following fish are farmed: trout (Oncorhynchus mykiss, Salmo trutta fario), char (*Salvelinus spp*.), sturgeon (*Acipenser spp*.), and eel (*Anguilla anguilla*).

5.1.2 Fish immunity

Teleost fish occupy a key position in the development of the innate and adaptive immune responses in that it is the earliest class of vertebrates possessing the element of both innate and adaptive immunity [261]. The teleost fish immune system includes most if not all of the elements of the innate immune system present in mammals. Moreover, taking into account the differences due to body compartments and cell organization, most of the secondary lymphoid organs present in mammals are also found in fish, except for lymphatic nodules and bone marrow [262].

The head kidney has hematopoietic functions [263] and unlike higher vertebrates it is the principal immune organ involved in phagocytosis [264], processing [265], production of IgM [266], and immune memory through melanomacrophagic centres [267], The main cells found in the head kidney of teleost fish are macrophages, which aggregate into structures called melanomacrophage centers (MMCs), and lymphoid cells, which are found at all developmental stages and exist mostly as Ig+ cells (B cells) [264].

The spleen is a large, blood-filtering organ that plays a vital role in haematopoiesis, antigen degradation and antibody production processing [268]. Moreover, Lefebvre F. *et al* have demonstrated that parasitic infections in fish are associated with a significant splenic enlargement and hyperfunction compared to uninfected fish. For this reason the spleen size of fish is widely used as a simple measurable immune parameter with a potential role in immune response against parasite infections [269].

Thymus is a lymphoid organ situated near the opercular cavity having a role in the production of T lymphocytes, in the stimulation of the macrophage phagocytosis and in antibody production by B cells [270]. At difference with mammals its involution in fish is more dependent on hormonal cycles and seasonal variations instead of the age.

The gut-associated lymphoid tissue (GALT) of teleosts consists principally of different lymphocytes, plasma cells and several types of granulocytes. Gut intraepithelial lymphocytes are largely considered T cells, whereas lymphoid cells present in the lamina propria are mainly B lymphocytes [271]. In bony fish, a very early (prethymic) appearance of T cells occur in the gut similar to extra-thymic origin of some T cells in mammals [272].

5.1.2.1 The adaptive immune system in fish

Fish are the most primitive vertebrates to possess adaptive immune system which includes lymphocytes, immunoglobulins, T cell receptor (TCR) and products of major histocompatibility complex (MHC) to allow the clonal selection of B and T cells. The teleost adaptive immune system also has other features, in addition to the aforementioned elements, which are similar to and in some cases differ from those of the mammalian immune system (Table 5.1).

Table 5.1 Fundamental features of adaptive immune systems of teleost fish and mammals

	Teleosts	Mammals
Immunoglobulin	IgM, IgD and IgT (or IgZ)	IgM, IgG, IgA, IgD and IgE
AID	Yes	Yes
Class-switch recombination	No	Yes
Somatic hypermutation	+++	+++
Affinity maturation	+	+++
Memory responses	+	+++
TCR, CD4, CD8	Yes	Yes
MHC class I and II	Yes	Yes
CD28, CD40, CD80, CD86, ICOS	Yes	Yes
T_H1, T_H2 and T_H17 cytokines	Yes	Yes
Spleen, thymus and bone marrow	Spleen and thymus but no true bone marrow	Yes
Mucosa-associated lymphoid tissue	Yes	Yes
Germinal centers and lymph nodes	No	Yes

Comparison of key elements of immunoglobulin-based adaptive immune systems of teleost fish and mammals. MHC, major histocompatibility complex; ICOS, inducible costimulator; TH1, TH2 and TH17, subsets of helper T cells. (Sunyer JO. Nature immunology. 2013)

The first evidence of the presence of T cells in teleosts was obtained *in vitro* using proliferation assays. Moreover, the increased availability of some tools has recently allowed the detection of some specific T cell markers [273]. For example, putative T cell markers in fish, such as CD3, CD4 and CD8a are known for a few species including atlantic salmon and turbot (*Scophthalmus maximus L*) [274,275]. T cells have also been characterized from the gut of rainbow trout, as they express transcripts of T cell marker homologs of CD8, CD4, CD28, CD3ε,TCR-ζ,TCR-γ,and TCR-β [276]. TCR gene expression is restricted to IgM- and IgT-negative lymphocytes in teleost fish [277].

B cells are characterized by the expression of B cell receptor, a surface immunoglobulin receptor (sIG). The expression of surface sIG-related receptors have been conserved in phylogenetically distinct species as a critical checkpoint in B cell development [278]. Fish B cells, like those of mammals, have been demonstrated to show Ig Heavy-chain rearrangement and allelic exclusion [279]. Fish have been shown to have lymphocyte subpopulations analogous to the mammalian B and T cells. Regarding the set of antibodies, it was shown that Ab repertoire in teleosts is

prevalently composed by IgM tetramer and furthermore is more limited compared to mammals [280]. IgM can be present in serum and secretions of fish, including cutaneous and gut mucus. The Ab response and serum concentration of IgM may vary between teleost species [272]. Recently a further two heavy chain isotypes have been identified, IgD and IgT, although they have not been completely characterized functionally [281]. IgD is thought to be located in the cell membrane of B cells, where it might act as a receptor. Another Ig class, IgZ was also reported in fish [273].

For lower vertebrate species like the teleost fish, the innate immune system is particularly critical for host survival in light of the poikilotermic nature plus the reduced repertoire of classical adaptive responses (limited antibody repertoires, affinity maturation and memory and relatively slow lymphocyte proliferation responses) compared to those of mammalian species [282].

5.1.2.2 The innate immune system in fish

The innate immune system of all multicellular organism is served by a variety of germline-encoded pattern recognition receptors (PRR). They are proteins expressed by cells of the innate immune system to identify pathogen-associated molecular patterns (PAMPs) such as peptidoglycans and lipopolysaccarides (LPS) in bacterial cell wall, fungal β-1-3 glucan, viral double stranded RNA and bacterial DNA. The PRR can be soluble components like the complement protein C3, lectins and various other humoral innate components or they can be expressed as receptors on phagocytes and other cells of the immune system [283]. There is evidence for β-1-3-glucan receptors on salmon macrophages [284] and on catfish neutrophils [285].

The Toll-like PRRs (TLRs) have also been described in fish. Key features of piscine Toll-like receptors and the factors involved in downstream signaling are structurally related to those of the mammalian TLR activation pathways. The structural conservation of the archaic TLR system suggests that also the regulation of the immune response might be similar in fish and mammals [286]. Like other vertebrate TLRs, also those of the teleost fish could be subdivided into six major families, each of which recognize a general class of molecular patterns [287]. Six non-mammalian TLRs were identified in fish. TLR 14 shares sequence and structural similarity with TLR1 and 2, and other five (TLR19, 20, 21, 22 and 23) form a cluster of novel TLRs. TLR4 was

lost from the genomes of most fish including rainbow trout, and the TLR-4 genes found in zebrafish do not recognize the mammalian agonist LPS and are likely paralogous and not orthologous to mammalian TLR-4 genes. TLR-6 and TLR-10 are also absent from all fish to date [288].

As in mammals, also the innate immune system of fish consists principally of three defensive elements: chemico-physical barriers, cellular elements and humoral elements.

Fish scales, mucous surfaces of skin and gills and the epidermis act as the first barrier against infection [289]. The important defence role of the mucus is well known and has been studied in several fish species [290,291]. Apart from efficient trapping of pathogens, fish mucus contains immune components like lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and IgM [292,293].

A broad selection of key cellular elements are involved in the non-specific cellular defense responses of teleost fish including non-specific cytotoxic cells (NCC), the neutrophilic granulocytes and other phagocytic cells such as monocytes/macrophages and dendritic-like cells [261] Some teleosts have been reported to have both acidophilic and basophilic granulocytes in peripheral blood in addition to the neutrophils, but in others only the latter morphological type has been found [294].

Non-specific cytotoxic cells (NCC) were the first identified and are the most extensively studied killer cell population in teleosts. Catfish NCC were found in the anterior kidney and spleen, and rarely in the peripheral blood (C.S. Tucker, J.A. Hargreaves. 2004). NCC kill a wide variety of target cells including tumour cells, virally transformed cells and protozoan parasites. Catfish and tilapia NCC have been found to express components of the granule exocytosis pathway of cell-mediated cytotoxicity similar to mammalian cytotoxic lymphocytes [295].

Similar to mammals, neutrophils are one of the first cell types to arrive at inflammatory sites and are a critical component of teleost innate immune defenses. This type of cells are equipped with a vast antimicrobial armamentarium used to limit the spreading of a broad range of pathogens including the production of reactive oxygen species [296] and nitric oxide [297].

As in mammals, monocytes/macrophages of teleost fish are phagocytic cells and possess potent intracellular antimicrobial defences. Both cell types have been shown to be variably capable of producing reactive oxygen, and nitrogen intermediates. For example, in goldfish monocytes a relatively short respiratory burst response and the

lack of nitric oxide response has been shown following activation [298], which could be compensated by the greater capacity of phagolysosome fusion compared to macrophages. To the other hand, goldfish macrophages produce a prolonged respiratory burst [299] and a potent nitric oxide responses [300] following activation, but have a more limited capacity for phagolysosome fusion. Based on this, it appears that monocytes may kill predominantly through phagolysosome fusion whereas macrophages may rely on respiratory burst and nitric oxide responses. Both this responses have been shown to be critical effector mechanisms in limiting the growth of fish pathogens [301]. Moreover, it has been shown that respiratory burst responses can be strongly activated by PAMPs. Among these compounds, LPS have focused many attention in the stimulation of respiratory burst responses in several fish species as goldifish [300], rainbow trout [302], Atlantic salmon and gilthead seabream (*sparus aurata*) [303].

Furthermore, other PAMPs with respiratory burst stimulation properties include polyinosinic:polycytidylic acid in rainbow trout [302], flagellin in gilthead seabream [303] and β-glucans including zymosan in rainbow trout [302] and *Saccharomyces cerevisiae* in gilthead seabream [304]. Finally, important pathogens of teleost fish have also been shown to induce potent respiratory burst responses. These include *Aeromonas* spp. in goldfish [305] and carp [306] and *Vibrio anguillarum* in gilthead seabream [307] infections.

Teleost fish possess also non-specific humoral defense substances with considerable physicochemical and functional similarity to those observed in mammals but with a number of different features.

An increasing number of AMPs have been isolated in fish and the high presence of these peptides in many tissues render them possible key player in the innate defence. Most are linear, amphipatic peptides that have been identified by virtue of their antimicrobial activity.

Many research groups have identified several antimicrobial peptides similar to mammalian cathelicidins in several teleost species but the immune properties of these compounds have not been fully understood also as a consequence of limited information about their function in the immune system. For example, Uzzell T. *et al.* have isolated three potent broad-spectrum antimicrobial peptides (HFIAP-1, -2, and -3) from intestinal tissue of *Myxine glutinosa* and have identified them as ancient members of the cathelicidin family [308]. Chang. CI. *et al*. have found three cathelicidin

genes in salmonids: two in atlantic salmon, named asCATH_1 and asCATH_2, and one in rainbow trout, named rtCATH_2 [51]. These salmonid cathelicidins share the common hallmarks of mammalian cathelicidin genes, such as a highly conserved preproregion and four invariant cysteines clustered in the C-terminal region of the cathelin-like domain. Maier VH. *et a*l. have identified several cathelicidin proteins in far related fish such as Atlantic cod (*Gadus morhua*) and Atlantic charr (*salvelinus alpinus*). They have also seen an upregulation of arctic charr and atlantic cod cathelicidins upon the infection caused by *Aeromonas salmonicida* suggesting a role of these proteins in fish innate immunity [309]. Scocchi M. *et al*. have investigated and characterized the cathelicidin gene family in three salmonids: brown trout (*Salmo trutta fario*), brook trout (*Salvelinus fontinalis*) and grayling (*Thymallus thymallus*) using genomic PCR amplifications and RT-PCR tissue analyses [52].

Another common group of AMPs in fish consist of piscidins, a family of peptides constituted by a highly conserved, histidin-rich, phenylalanine-rich N-terminus and a more variable C-terminus with a potent and broad-spectrum of activity against several micro-organisms [310]. First isolated from mast cell of hybrid see bass, piscidins have recently been localized to another immune cell, the acidophilic granulocyte which is the most abundant circulating phagocytic granulocyte and considered functionally equivalent to the neutrophil of higher vertebrates [311] Four piscidin from hybrid stripped bass were active in the reduction of infectivity caused by channel catfish virus (CCV) and frog virus 3 (FV3) [312]. Piscidin 1 and -3 possess antimicrobial activity against several important bacterial fish pathogens such as *Streptococcus iniae*, *Lactococcus garviae*, *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Vibrio alginolyticus* [310]. Moreover, these type of AMPs have also shown fungicidal and antiparasitic activity [313].

Aside from antimicrobial peptides, all eukaryotic organisms, including fish cells, produce a diverse array of natural anti-infective agents that include proteins and other molecules often not related to the innate defences. Examples are histones and their polypeptide fragments. Proteins highly homologous to or identical to core nuclear histones have been identified from a number of fish. Histone-like proteins (HLPs) were originally isolated from the skin of channel catfish [314] and subsequently identified in skin, gill and/or spleen of hybrid stripped bass and rainbow trout (*Onchorynchus mykiss*) [310]. The presence of histone-related AMPs in a wide range of fish species suggests that they have a role as a host defense in most if not all teleosts. This is also

supported by results of *In vitro* studies demonstrating that HLPs, as well as their peptide fragments, are active against many important fish pathogens, including bacteria Vibrio *anguillarum*, *Vibrio salmonicida*, *Vibrio alginolyticus*, *Aeromonas Hydrophila* and *Aeromonas salmonicida* [314].

The complement system of teleost fish, like that of higher vertebrates, can be activated through the three pathways of complement. The classical pathway is triggered by binding of antibody to the cell surface but can also be activated by acute phase proteins such as ligand-bound C-reactive protein or directly by viruses, bacteria and virus-infected cells. The alternative pathway is independent of antibody and activated directly by foreign microorganisms and the lectin pathway is elicited by binding of a protein complex consisting of mannose-binding lectins to mannans on bacterial cell surfaces [315]. All three pathways finally converge to the lytic pathway, leading to opsonisation or direct killing of the microorganism. Fish complement, in general, exhibits highest activity between 15 °C and 25 °C and can remain active at temperatures as low as 0–4 °C which is in contrast to mammalian complement with an optimal temperature of 37 °C [316]. It is likely that this lower and wider range of optimum temperature, compared to mammalian complement, allows non specific fish immune functions to operate adequately to compensate the slow or suppressed adaptive immune response observed at low temperatures [317].

The hemolytic activity of serum is considered as a function of complement activity by the alternative pathway [315] and it has been suggested that some of the differences between mammals and teleosts indicate a greater importance of the alternative pathway in the innate response of teleosts compared to mammals or a species-specific difference in the sensitivity of target molecules [261].

Lectins (haemagglutinins) are proteins of non-immune origin that agglutinate cells and precipitate glycoconjugates. Their ability to bind to terminal sugar on glycoproteins makes them important pattern recognition receptors in innate immunity. Apart the involvement in lectin complement pathway, it has been show that lectins could also act as opsonins for phagocytosis of bacteria [318].

Natural antibodies are produced at tightly regulated levels in the complete absence of exogenous antigenic stimulation [319]. They provide immediate, early and broad protection against pathogens, making them a crucial non-redundant component of the innate humoral immune system. Natural antibodies have been identified in the sera of normal, non-immunized humans, mice and some teleosts [320].

In recent years, the study of fish cytokines and chemokines has generated big interest leading also to a significant progress in the isolation of these molecules from fish [261]. The identification of fish cytokines is hampered by low sequence identity compared to their mammalian counterparts. In general, however, fish appear to posses a repertoire of cytokines similar to that of mammals. To date several cytokine homologues have been cloned in fish species, including tumour necrosis factor-α (TNFα) and TNFβ, interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, IL-11, IL-12, IL-15, IL-18, IL-21, IL-22, IL-26, IFN-γ and several chemokines such as IL-8 or CXCL8, γIP-10, CK-1 and CK-2 [261].

5.1.3 Commercial immunostimulants in aquaculture

Various kinds of substances have been studied for their capacity to produce immunomodulatory effects, but only few of them have demonstrated to be useful in aquaculture.

In recent years, attention has been focused on the use of β-glucans in fish. The term “glucan” is used generically to describe a group of glucose polymers, consisting of a backbone of β (1, 3)-linked β-D-glucopyranosyl units with β-(1, 6)-linked side chains of varying distribution and length. In nature, β-glucans are present in the cell wall of many plants (wheat, rye, barley, and oat), baker’s and brewer’s yeast (Saccharomyces genus), and Echinaceae members [321]. Many studies have been carried out on different fish species such as Atlantic salmon [322], rainbow trout [323,324], Snapper [325], African catfish [326], Prawn [327] and Sea bass [328] showing a role of β-glucan on the growth [325], survival, resistance, and protection against pathogen [329] antibody production [330], immune-related gene expression [331], and as adjuvant [332] in a wide range of fish species. Generally, β-glucan is used as feed ingredient in fish diet.

Besides β-glucans also a seaweed-based meal rich in alginates and polysaccharides called Ergosan has found successful commercial application as immunostimulant. A single dose of 1 mg of Ergosan significantly augmented the proportion of neutrophils, increased the degree of phagocytosis, respiratory burst activity and expression of interleukin-1β (IL-1β), interleukin-8 (IL-8) and one of the two known isoforms of trout tumour necrosis factor-alpha (TNF-α) in peritoneal leukocytes of rainbow trout at 1 day post-injection [333]

Other important immunostimulants in aquaculture are nucleotides that have attracted attention due to their capacity to modulate lymphocyte maturation, macrophage phagocytosis, immunoglobulin responses and genetic expression of certain cytochines [334,335]

5.1.4 AMPs as immunostimulants

The role of AMPs in the regulation of immune responses in mammalian species is currently well known. To the other hand, little is known about the effects of these peptides in fish. For example Chiou PP. *et al*. have shown that three linear cationic alpha-helical antimicrobial peptides (insect cecropin B, fish pleurocidin and cecropin analogue CF17) are able to induce an over expression of interleukin-1 beta and cyclo-oxygenase-2 in the rainbow trout cell line RTS11 [336]. Bridle A. *et al*. have demonstrated that salmon cathelicidins 1 and 2 (asCATH1 and asCATH2) stimulate peripheral blood leukocytes increasing the transcription of the chemokine interleukin-8 [337]. Other few peptides were also studied for their immunomodulatory effects in fish, including the human alpha defensin-1 (HNP1). This peptide was able to up regulate genes related to the type I interferons (IFN) in trout head kidney leukocytes [338].

As a first step to evaluate the potential of AMPs as immunostimulants to prevent aquaculture infections, we have analyzed the effects of selected AMPs on the respiratory burst and on the expression of pro-inflammatory cytokine genes in head kidney leukocytes from rainbow trout. The latter is one of the most economically important fish species in Friuli Venezia Giulia region providing about 40% of rainbow trout italian demand.

5.2 MATERIALS AND METHODS:

5.2.1 Fish and experimental conditions

Adult rainbow trout (*O. mykiss*), mean weight 300-400 g, were obtained from the fish facilities of the Department of Food Sciences (University of Udine). Fish were acclimatized and held in a 3000 l fibre-glass tank supplied with spring water at 11 ± 2°C, under natural photoperiod. During the experimental phase they were fed with a commercial diet at a level recommended by the manufacturer (Veronesi, Italy) and were kept under veterinary control. The fish have been always handled under sedation and the experimental procedures were performed in a way to minimize suffering and pain. Cells from a single fish were followed throughout the experiment and comparison was done among individual fish following treatments with peptides and various stimulants. Thus, cells from individual fish were not pooled but maintained as individual cell culture throughout the entire experiment.

5.2.2 Head kidney leukocytes

Head kidney leukocytes (HKL) were isolated and cultured according to the methods previously described Chettri JK. *et al*. with minor modifications [341]. Briefly, the head kidney was aseptically removed and placed in a Petri dish containing cold RPMI-1640 with 12,5 UI/ml heparin. Small pieces of head kidney were squeezed using a plunger of a 5 ml syringe. The resultant cell suspension was collected without the head kidney sediments and was subsequently centrifuged (200 g for 10 min; 4°C). After centrifugation, the supernatant was removed, the cell pellet was resuspended in RPMI-1640 with 12.5 UI/ml heparin and layered onto Histopaque-1077 (d = 1.077 g/ml) solution in a sterile centrifuge tube. After centrifugation at 300 g for 25 min; 4°C, cells at the interface were collected and washed twice with sterile Hank's balance salt solution (HBSS) (300 g for 10 min; 4°C). Cell viability was determined with the trypan blue exclusion method and the cell concentration was adjusted to $2.5x10^6$ cells/ml in HBSS medium containing 0.125 % (w/v) bovine serum albumin (HBSS-BSA) for respiratory burst experiments. Alternatively the obtained HKL were resuspended in Leibovitz medium (L-15) supplemented with 10% FCS and antibiotics (penicillin-streptomycin) at concentration of $1x10^7$ cell ml^{-1} for Real time-PCR experiments.

5.2.3 Cultivation of RTG-2 cell line

The RTG-2 cell line, a fibroblast-like cell culture derived from gonad of normal rainbow trout (*Oncorhynchus mykiss*) [339], was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna. It was maintained at 20°C in 25- or 75-cm^2 tissue culture flasks in L-15 medium supplemented with 10% Foetal calf serum (FCS), glutamine and antibiotics (penicillin-streptomycin). Culture at 70-80% confluence (2-3 $x10^6$ cells) were splitted weakly by detaching cells with 2 ml per flask of 0.25% trypsin-EDTA solution. Detached cells were added with complete L-15 medium to inactivate trypsin, and then diluted at $1,5x10^6$ cells/ml for subsequent culturing or at $5x10^5$ cells/ml for cytotoxicity experiments.

5.2.4 Peptides.

The salmonid cathelicidin STF and the bovine cathelicidin BMAP-28 were chemically synthesized according to standard methods. The good quality of crude peptides were confirmed by electrospray ionization-mass spectrometry (ESI-MS) (Esquire 4000, Bruker Daltonics Inc., Billerica, MA, USA). STF concentration was measured using the Waddell method, whereas the concentration of BMAP-28 was determined by measuring the absorbance at 280 nm, taking into account the extinction coefficients of Trp and Tyr (6839 cm^{-1} M^{-1} at 280 nm).

5.2.5 Other reagents

Lipopolysaccharide from Escherichia coli 0111:B4 (LPS), β-Glucan (from baker's yeast, *Saccharomyces cerevisiae*) and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich, resuspended in sterile apyrogenic water and mantained at -20°C (PMA) or 4°C (LPS and β-glucan) until use.

5.2.6 Cytospin Preparations

Cytospin preparations of isolated leucocytes at $1-2x10^5$ cells ml^{-1} in complete L-15 medium were prepared by centrifugation at 200 g for 10 min in a cytospin apparatus. The smears of cells were air dried at room temperature, fixed with methanol (100%) and stained with Giemsa. The slides were examined with a coverslip under oil immersion objective with an optical-microscope (Leica DMRB) at the magnification of 1000X (100x x 10x).

5.2.7 Measurement of respiratory burst activity

The production of ROS was measured by real-time luminol enhanced chemiluminescence according to Vera-Jimenez NI. *et al*. [340] with minor modifications. Black flat-bottomed 96-well microtiter plates were prepared containing freshly purified HKL at $2.5x10^6$ cells/well and 0,5 mM luminol in HBSS-BSA in a final volume of 100 μl/well. The production of ROS was triggered by the addition of: 100 μl of 1 μg/ml PMA or 250 μg/ml β-Glucan; 5 μl peptide-containing solution at different concentrations; 100 μl of 1 μg/ml PMA or 250 μg/ml β-Glucan plus 5 μl peptide-containing solution. Control wells were added with 100 μl HBSS. Each sample was run in triplicate. The reaction was monitored for 20-40 min in a microplate luminometer (Sunrise TECAN; integration time 0,5 s, photomultiplayer gain = 180) every 2 min at room temperature. The results are reported as relative light units (RLU) recorded during the incubation periods. For quantification and statistical analysis of data, fold increase values were calculated as the ratio of $RLU_{combination}/(RLU_{peptide} + RLU_{stimulant})$.

5.2.8 Real-time PCR quantification.

RNA was isolated from head kidney leukocytes samples using Illustra RNAspin (GE Healthcare Europe). and treated with DNase I (DNA-free, Apllied Biosystem/Ambion, Austin, TX, USA), according to the manufacturer's instructions. One microgram of total RNA was incubated for 5 min at 70°C with oligo(dT) and random primers. After this incubation period 200 units of Superscript II reverse transcriptase (Invitrogen) and 40 units of RNaseOUT were added to each sample and the resulted solution was incubated 10 min at 25°C, 50 min at 40°C and 10 min at 70°C. Quantitative real-time PCR (qPCR) was carried out for each sample in triplicate using the SYBR green fluorescent detection system and the iCycler iQ5 (Bio-Rad Laboratories, Segrate, Italy). Each reaction mixture (15 μl) contained 7.5 μl SYBR green Master Mix (Bio-Rad Laboratories), 0.45 μl each primer at 10 μM, 5.1 μl water, and 1.5 μl of a 5-fold-diluted cDNA product. The thermal-cycling program was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Following qPCR cycles, dissociation curves were run to assess the presence of only one product and the absence of primer dimers. Primers for *O. mykiss* tumor necrosis factor alpha (TNF-α), interleukin 1-β (IL-1β) and the reference 60S ribosomal protein L31 (Table 5.1) were as previously reported by Perez-Sanchez T. et al. 2011. Primers for interleukin-8 (IL-8) (Table 5.1) were previously reported by Montero J. *et al.* [360]. The abundance

of specific gene transcripts was normalized in each sample relative to ribosomal gene 60S, and the results were expressed as normalized fold expression relative to the control (untreated).

Table 5.1 Primer pairs used in qPCR

Target cDNA	Oligonucleotides (5'-3')[a]	Product size (bp)	Accession no. (GeneBank)
IL-1β	F: ACATTGCCAACCTCATCATCG R: TTGAGCAGGTCCTTGTCCTTG	91	AJ223954
IL-8	F: ATTGAGACGGAAAGCAGACG R: CTTGCTCAGAGTGGCAATGA	105	AJ279069
TNF-α	F: GGGGACAAACTGTGGACTGA R: GAAGTTCTTGCCCTGCTCTG	75	AJ277604
60 S	F: AGCCACCAGTATGCTAACCAGT R: TGTGATTGCACATTGACAAAAA	147	NM001165047

[a]F, forward; R, Reverse

5.2.9 Assessment of membrane permeabilization

To assess the plasma membrane integrity of peptide-treated HKL, the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the medium was measured in the conditions used for real time experiments (adherent HKL) and for respiratory burst analysis (suspended HKL) by using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega). In addition, the effects of the peptides on plasma membrane integrity was evaluated also in RTG-2 cells, which are commonly used for toxicology studies.

Conditions used in Respiratory Burst experiments (Suspended HKL). Cell suspensions at a density of $2.5x10^6$ cells/ml in HBSS-BSA, were incubated in the absence and in the presence of different peptide concentrations for 1 h at 18 °C in 96-well tissue culture plates. After centrifugation at 250 g for 5 min the LDH activity was measured spectrophotometrically at 490 nm in supernatants and cell lysates.

Conditions used in Real-Time experiments (Adherent HKL). 100 µl of $1x10^7$ cells/ml in complete L-15 medium were seeded overnight at 18°C in 96-well tissue culture plates. After this period adherent cells were treated 1h at 18°C in the absence and in presence of different peptide concentrations. After centrifugation at 250 g for 5

min the LDH activity was measured spectrophotometrically at 490 nm in supernatants and cell lysates.

RTG-2 cell line. 100 µl of $5x10^5$ cells/ml in complete L-15 medium were seeded overnight at 22°C in 96-well tissue culture plates. After this period adherent cells were treated 1h at 22°C in the absence and in the presence of different peptide concentrations. After centrifugation at 250 g for 5 min the LDH activity was measured spectrophotometrically in supernatants and cell lysates.

Lactate dehydrogenase activity in the culture media was expressed as percentage of total cellular lactate dehydrogenase activity.

5.2.10 Statistical Analysis

Statistical differences among groups of data were analysed by one-way analysis of variance followed by the Bonferroni post test, using GRAPHPAD Prism version 5.0. In all comparisons, $P<0.05$ was considered significant.

5.3 RESULTS:

5.3.1 Peptides under study

The sequence of the peptide STF used in this study was derived from the C-terminal region of the fish cathelicidin CATH1_SALTR identified in brown trout (*salmo trutta*) by [52]. The salmonid cathelicidins are characterized by the typical four-exon structure shared by the cathelicidin genes [28] with a very conserved sequence of the exons 1- 3 and a highly variable amino acid sequence corresponding to exon 4. Based on the comparison of the reported amino acid sequences encoded by exon 4, they are highly cationic, with a net charge between +11 and +17 and possess a high content of Gly (25-31%) and Ser (8-21%) residues [52]. Unpublished results indicate a variable activity of STF against gram positive and gram negative fish pathogens, whereas the immunomodulatory properties of this peptide have not yet been characterized. In this study, we investigated the effects of this peptide on the induction of the respiratory burst and on the cytokine gene expression in primary HKL, in comparison to the bovine cathelicidin BMAP-28, an antimicrobial peptide endowed also with immunomodulatory properties [76].

5.3.2 Head kidney Leukocytes (HKL)

The kidney in teleost fish is the equivalent of the bone marrow in vertebrates and is the largest site of haematopoiesis [264]. Head kidney leukocytes (HKL) are a central element in a number of *in vivo* and *in vitro* assays elucidating innate and adaptive immune mechanisms in teleosts following stimulation with various antigens [341]. We thus decided to use freshly purified HKL to investigate the immunomodulatory properties of the peptides under study. Since different cell types are present in HKL preparations, we analyzed the morphology of the HKL immediately after the purification procedure and found they mainly consisted of macrophages (35%), lymphocytes (29%), monocytes (27%), neutrophils (2%) (Figure 5.1A, B, C, D respectively) and 5% of undefined cells. The only reference data available to date for rainbow trout are that reported by Velisek J. *et al*. for rainbow trout peripheral blood leukocytes [lymphocytes (75%), monocytes (3%), neutrophils (21%), developmental phases (1%)] [359]. However, the comparison between these two leukocytes populations may be only indicative due to the biological and experimental variability

between the two organs and between different fish subjects. Noteworthy, it is reported that head kidney cells incubated for several days in culture exhibit the cytological characteristics of macrophages [342].

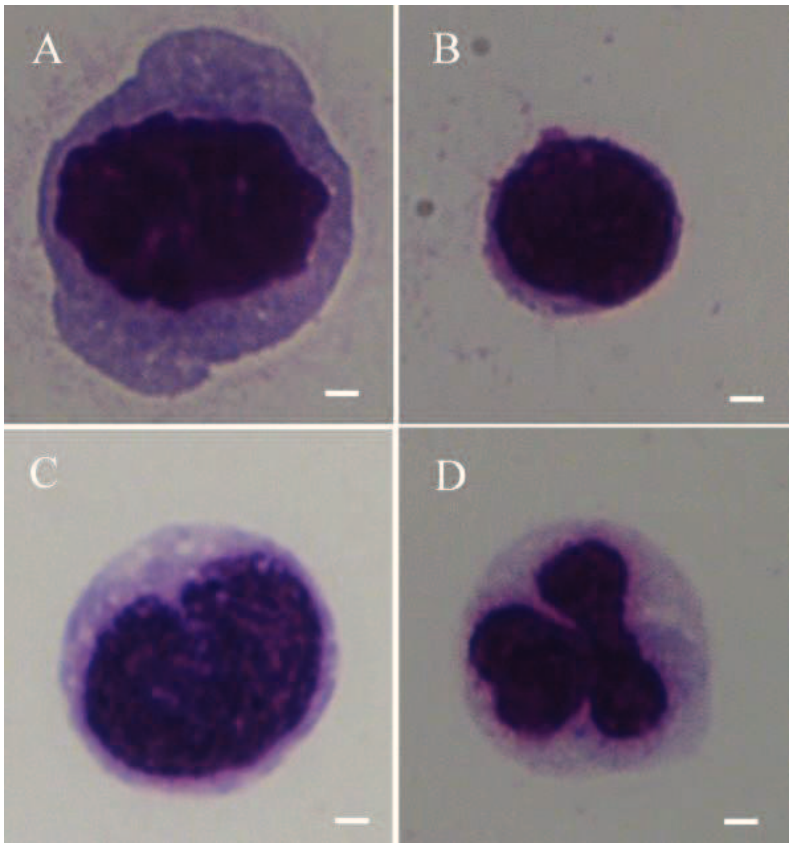

Figure 5.1 Light microscopic photographs (bars 1μm) of representative cell types derived from cytospin preparations of freshly isolated head kidney leukocytes fixed with methanol and stained with Giemsa: (A) macrophages, (B) lymphocytes, (C) monocytes and (D) neutrophils.

5.3.3 Lactate dehydrogenase release (LDH)

To evaluate the membrane permeabilizing activity of the peptides under study, the release of the cytoplasmic enzyme lactate dehydrogenase in the medium was measured after 1 h of incubation in the presence and in the absence of the peptides, in freshly suspended and in adherent HKL (Table 5.2). Moreover, the cytotoxic activity of the peptides was also evaluated on the fibroblast-like RTG-2 cell line derived from rainbow trout gonadal tissue [339] which is widely used for ecotoxicity studies [343-345]. None of the peptides under study caused a significant release of LDH in the culture medium. Only 4 μM BMAP-28 produced about 8-10% LDH release in adherent HKL and RTG-2 cells, respectively. It is interesting to note a general slight increase in the % of LDH release in suspended HKL in comparison to the adherent cells. This could be a consequence of several factors including the more stressed condition of the freshly purified cells and a reduced percentage of serum in the medium.

Table 5.2 Lactate dehydrogenase release in peptide treated cells

	Adherent HKL	Suspended HKL	RTG-2 cell line
Untreated cells	0.72 ± 0.37	1.88 ± 0.85	0.40 ± 0.33
STF 4 μM	1.05 ± 0.30	1.91 ± 0.67	0.67 ± 0.38
STF 8 μM	1.12 ± 0.66	1.95 ± 0.73	0.85 ± 0.77
STF 16 μM	1.30 ± 0.30	3.94 ± 2.50	1.40 ± 0.84
BMAP-28 2 μM	2.72 ± 1.28	2.80 ± 0.54	1.14 ± 0.31
BMAP-28 4 μM	8.12 ± 0.85	12.59 ± 3.08	10.00 ± 1.75
β-Glucan 125 μg/ml	2.11 ± 0.74	3.63 ± 0.63	0.59 ± 0.58
LPS 100 μg/ml	3.08 ± 0.55	4.45 ± 0.72	0.63 ± 0.38
PMA 0.5 μg/ml	1.92 ± 0.84	3.02 ± 0.77	-

The results are expressed as % LDH activity of the supernatants with respect to total LDH activity and are the mean ± SD of three independent experiments performed in duplicate.

5.3.4 Production of reactive oxygen species

Phagocytes such as macrophages and neutrophils play an important role in limiting the dissemination of infectious agents, and are responsible for the eventual destruction of phagocytosed pathogens [298]. Moreover, it is well established that fish macrophages can be activated *in vivo* and *in vitro* for enhanced bactericidal activity [346-348]. As early as within the first minutes of stimulation, phagocytic cells release large quantities of highly toxic reactive oxygen species, during the so-called "respiratory burst." ROS are known to belong to the most efficient microbicidal mechanisms. Thus, phagocytic cells represent the front-line defence cells in protecting organisms against infection and play an irreplaceable role in the proper performance of the immune system [349].

Since HKL are prevalently composed by phagocytic cells, we asked whether the peptides under study have the ability to stimulate the respiratory burst in these cells. In this regard, the ability of BMAP-28 and STF to stimulate ROS production in HKL, freshly isolated from different fish subjects, was measured by using a luminometric method. Moreover, the combination of each single peptide with the well known stimuli phorbol-12-myristate-13-acetate (PMA) or β-glucan was also tested to study the possible adjuvant ability of the peptides on the induction of respiratory burst.

Due to experimental variability in respiratory burst responses among different subjects, representative experiments are reported in Figures 5.2 and 5.3.

0.5 μg/ml PMA (Figure 5.2 A) and 125 μg/ml β-glucan (Figure 5.3 A) were able to induce ROS production in HKL with different potencies and different kinetics, when the stimuli were added alone. 0.5 μg/ml PMA gave rise to a rapid ROS production, that reached the maximum at 10 minutes (Figure 5.2 A), whereas 125 μg/ml β-glucan needed about 28 min to reach the maximum (Figure 5.3 A). These differences in the ROS production kinetics between the used stimuli are a consequence of their different mechanisms of activation of the respiratory burst. In leukocytes, PMA enhances ROS production via a direct protein kinase C (PKC)-dependent activation of NAD(P)H oxidase [350] whereas β-glucan requires the initial binding to the membrane receptor dectin-1 inducing intracellular signalling that leads to the production of ROS via a Syk kinase-dependent activation of protein kinase C [351].

Concerning the peptides under study, 2 μM STF (Figure 5.2 C and Figure 5.3 C), but not BMAP-28 (Figure 5.2 B and Figure 5.3 B), was able to induce a slight ROS increase that reached the maximum at 4 min when the peptide was added alone. Importantly, both BMAP-28 and STF produced a synergistic ROS production when they were added in combination with PMA (Figure 5.2 D and Figure 5.2 E, respectively) or β-glucan (Figure 5.3 D and Figure 5.3 E, respectively), but with different kinetics. Specifically, BMAP-28 increased the intensity of the respiratory burst in both, PMA- and β-glucan-stimulated cells, without affecting the kinetics of the burst responses produced by each of these stimuli alone. On the contrary, STF produced a stronger synergistic effect at earlier time points in comparison to that produced by BMAP-28, reaching the maximum value between 4 and 6 min in PMA- and 8 min in β-glucan- stimulated cells. Interestingly, the combination of STF and PMA or STF and β-glucan induced the maximum ROS production at earlier time points with respect to that induced by PMA (Figure 5.2 E, open bars *vs* closed bars) or by β-glucan (Figure 5.3 E, open bars *vs* closed bars) when these stimuli were added alone.

In order to quantify the synergistic effects observed and to statistically analyze the data obtained with HKL from different fish subjects, results of different experiments were calculated as "fold increase" according to [303,352] with some modifications. Fold increase values were calculated as the ratio of $RLU_{combination}/(RLU_{peptide} + RLU_{stimulant})$ and the mean values of three independent experiments are reported in Figure 5.4.

According to these calculations, a combined effect of two stimuli would be considered synergic and not additive at fold increase values higher than one. Indeed, a maximum of about 4-fold increase at 2 min (Figure 5.4 B) and 3.5-fold increase at 4 minutes (Figure 5.4 D) in ROS production was observed when STF was used in combination with PMA or β-glucan, respectively. On the other hand, the slight increase produced by BMAP-28 in combination with PMA did not reach statistical significance (Figure 5.4 A).

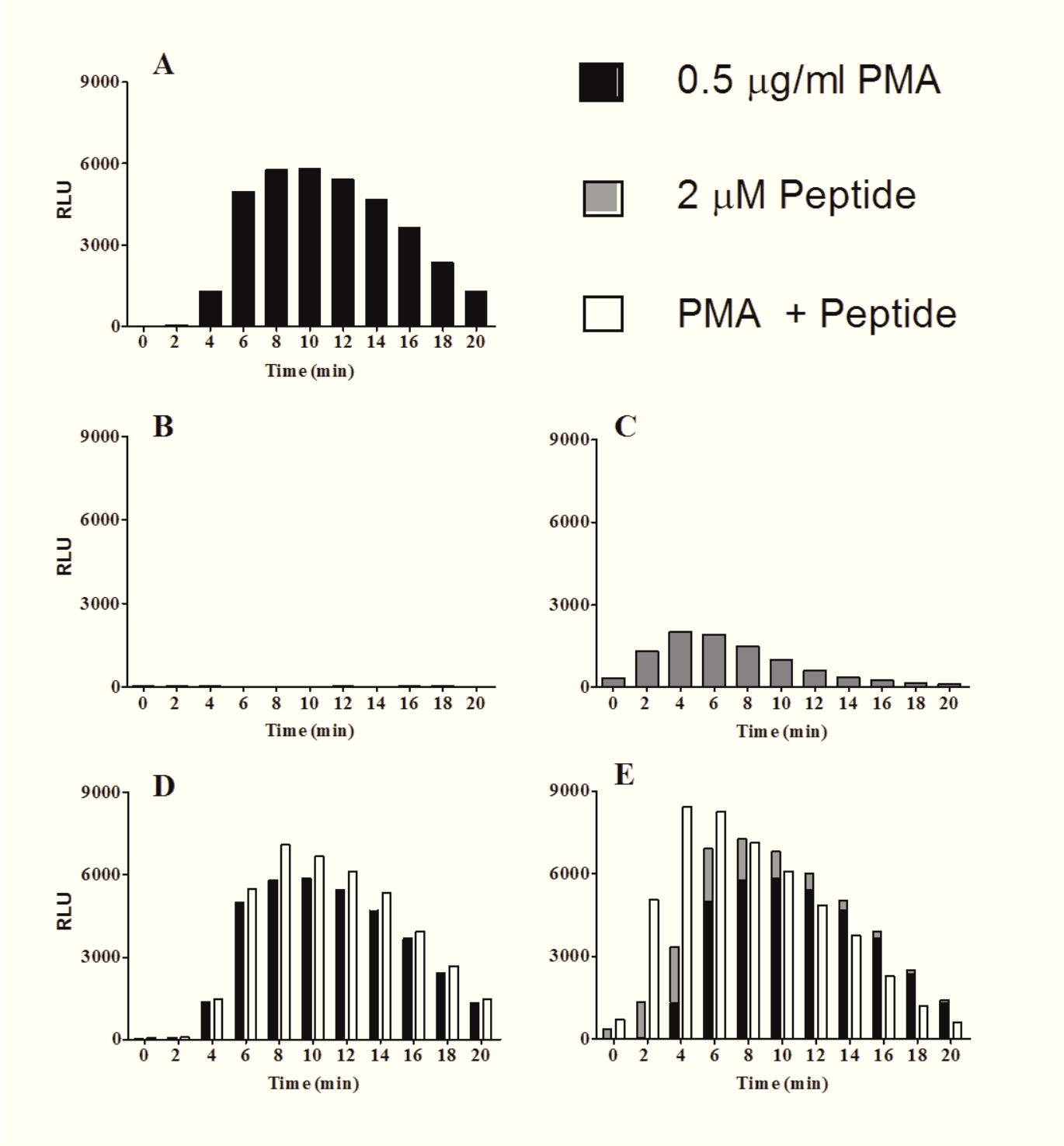

Figure 5.2 ROS production in HKL stimulated by PMA and AMPs.
Time course analysis of ROS production triggered by 0.5 μg/ml PMA (A), 2 μM BMAP-28 (B), 2 μM STF (C) and the combinations of 0.5 μg/ml PMA plus 2 μM BMAP-28 (D) and 0.5 μg/ml PMA plus 2 μM STF (E) as determined by luminometric analysis. Data are expressed as relative luminescence unit and are representative of one out of three independent experiments showing similar results.

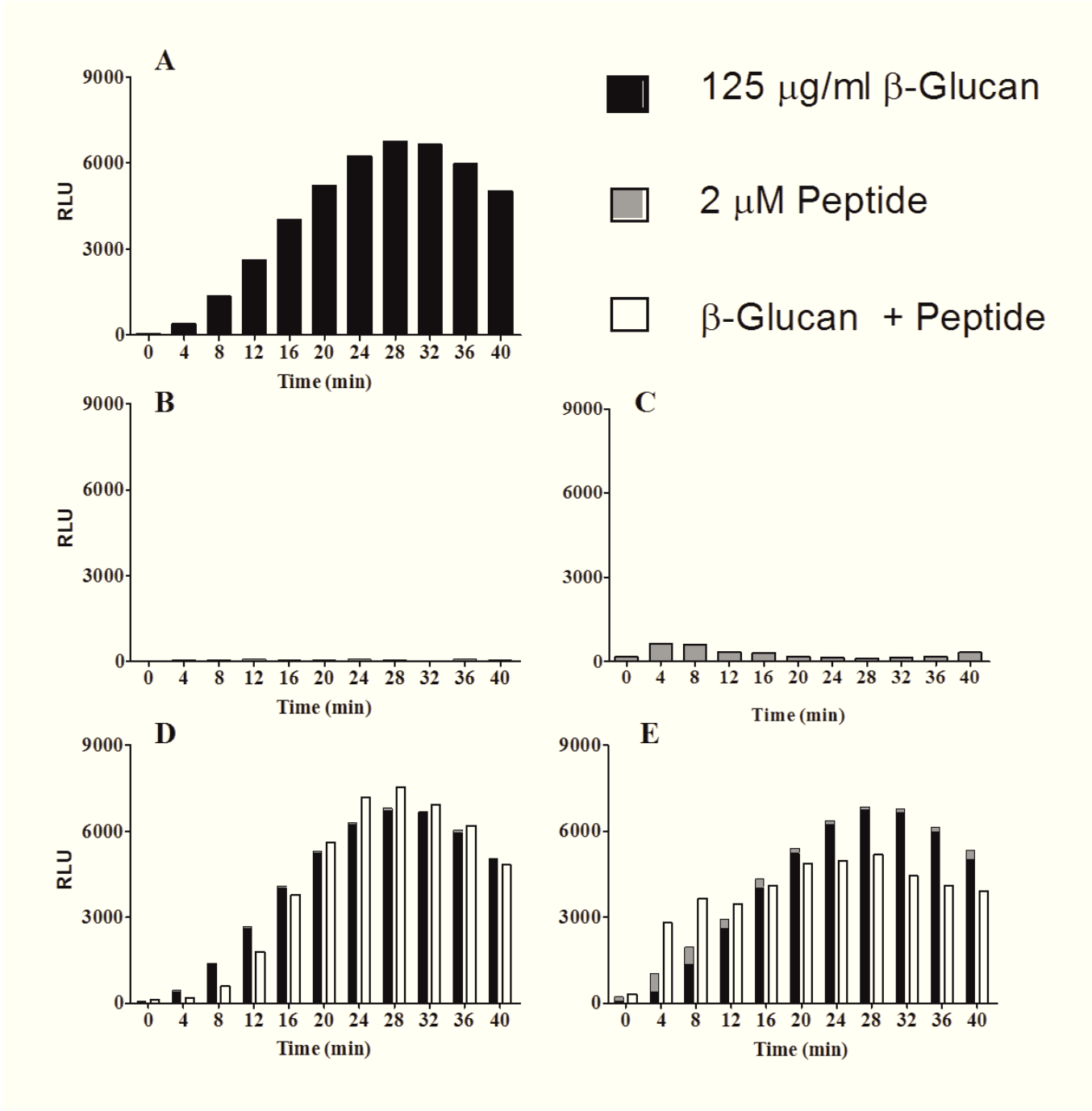

Figure 5.3 ROS production in HKL stimulated by β-glucan and AMPs

Time course analysis of ROS production triggered by 125 μg/ml β-glucan (A), 2 μM BMAP-28 (B), 2 μM STF (C) and the combinations of 125 μg/ml β-glucan plus 2 μM BMAP-28 (D) and 125 μg/ml β-glucan plus 2 μM STF (E) as determined by luminometric analysis. Data are expressed as relative luminescence unit and are representative of one out of three independent experiments showing similar results.

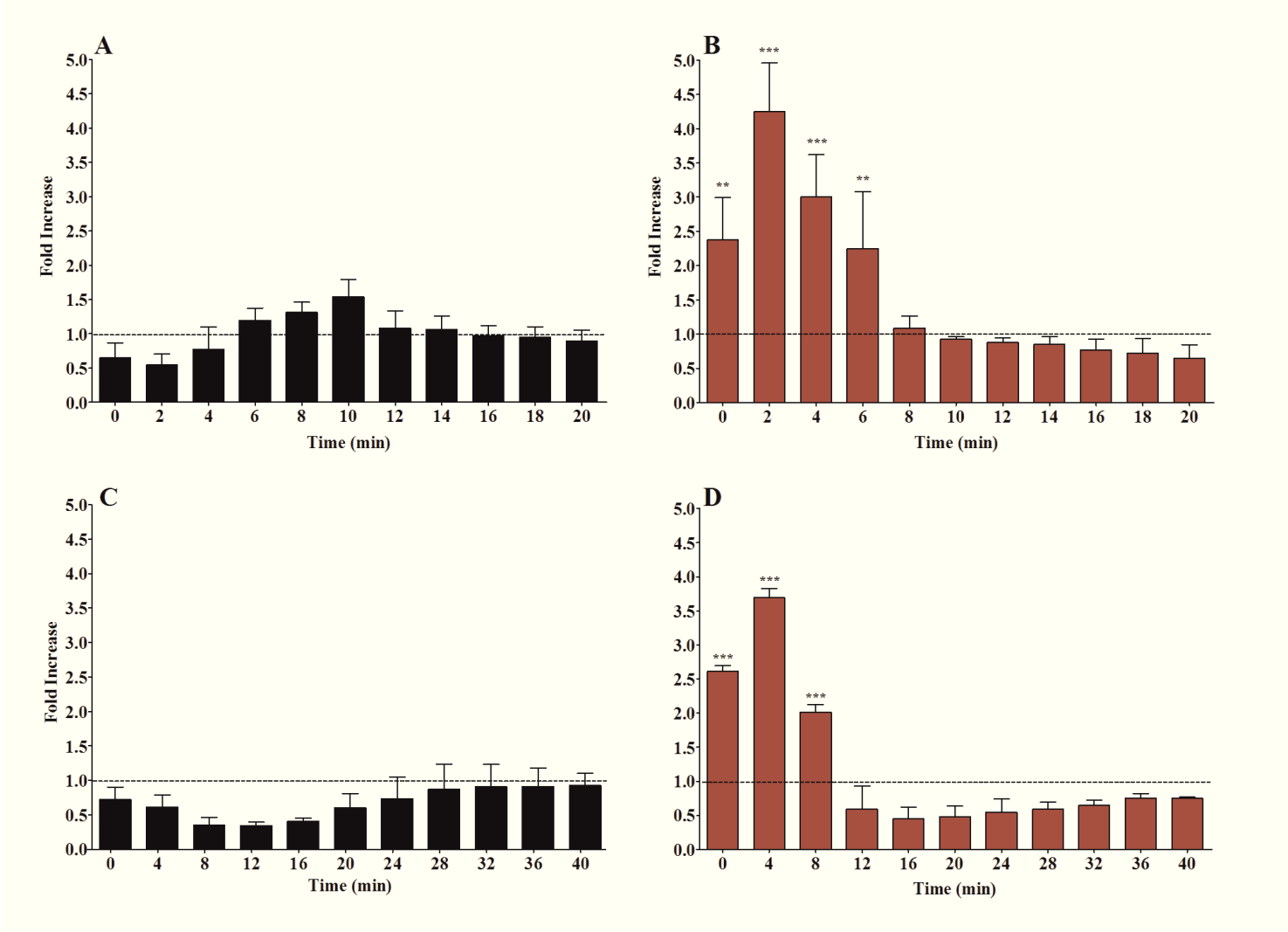

Figure 5.4 Effect of the combination of AMPs and stimulants (PMA or β-glucan) on ROS production in HKL.

Time course analysis of ROS production triggered by 0.5 μg/ml PMA (A and B); 125 μg/ml β-glucan (C and D); 2 μM BMAP-28 (A and C); 2 μM STF (B and D); the combination of 0.5 μg/ml PMA plus 2 μM BMAP-28 (A); 0.5 μg/ml PMA plus 2 μM STF (B); 125 μg/ml β-glucan plus 2 μM BMAP-28 (C); 125 μg/ml β-glucan plus 2 μM STF(D). The effects of the combination of AMPs and stimulants are expressed as "fold increase" values that were calculated as the ratio of $RLU_{combination}/(RLU_{peptide} + RLU_{stimulant})$. Values are the mean ± SD of three independent experiments performed in triplicate. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

5.3.5 Cytokine gene expression in HKL

As the next step in the evaluation of the immunomodulatory properties of the two peptides under study we asked whether these molecules could be able to modulate the pro-inflammatory cytokine gene expression in HKL. Our hypothesis was supported by published evidence indicating that atlantic salmon cathelicidin 1 and 2 were able to stimulate peripheral blood leukocytes increasing the transcription of the chemokine interleukin-8 [337]. Moreover, D'este F. *et al*. have seen that BMAP-28 was able to modulate cytokine gene expression in murine macrophages in addition to its capacity to alter the LPS-mediated inflammatory response through direct effects on macrophages [76].

In these experiments, isolated HKL were seeded overnight in a microtiter plate to allow cell adhesion at 18 °C in L-15 complete medium. After this period, non adherent cells were removed and adherent cells were exposed to BMAP-28, STF and two $PAMP_S$ such as LPS and β-glucan, mimicking bacterial and fungal infections, respectively. LPS and β-glucan were used due to their reported ability to activate HKL in terms of cytokine gene expression [341]. Moreover, each peptide was added to adherent HKL either alone or in combination with LPS or β-glucan. After a incubation period ranging from 1-8h, the expression profiles of three immune relevant genes (IL-1β, IL-8 and TNF-α) were determined by qRT-PCR.

As reported in Figure 5.5, the expression of IL-1β increased significantly following exposure to 100 μg/ml LPS. This gene showed a time dependent response with highest up regulation after 8 h of exposure. TNF-α and IL-8 expression showed a similar pattern after stimulation with LPS with highest up regulation after 4h. Similarly, exposure of adherent HKL to 125 μg/ml β-glucan led to a significant up-regulation of IL-1β and IL-8 at 4h that was higher compared to that of LPS at the same time point. To the other hand, no significant change in the TNF-α expression was observed after stimulating HKL with 125 μg/ml β-glucan. Importantly, all these results are in line with those obtained by Chettri KJ. *et al*. in HKL [341].

The gene expression of the three selected cytokines was not significantly affected by STF up to 32 μM and BMAP-28 up to 4 μM up to 8 h of incubation (not shown). However, combination of 2 μM STF with 125 μg/ml β-glucan produced a slight synergistic effect in IL-1β expression without statistical significance (not shown).

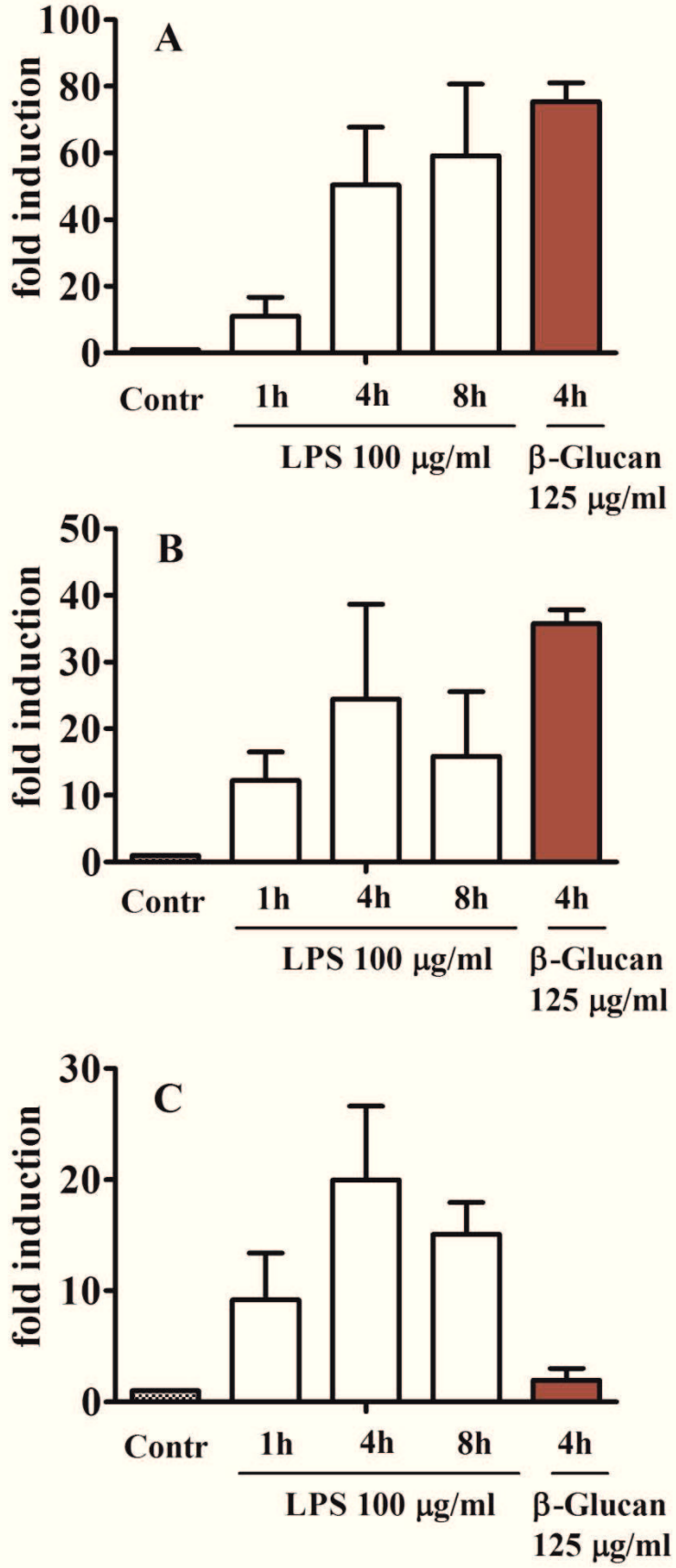

Figure 5.5 Expression of A) IL-1β, B) IL-8 and C) TNF-α (as fold induction) compared to time point controls after exposing adherent HKL from rainbow trout to different molecular patterns.

5.4 DISCUSSION:

Aquaculture is as an economic activity of increasing importance for the global food supply. However, the sustainable development of aquaculture is facing a number of problems. Among them, infectious diseases of diverse aetiology are of particular importance as the intensification of aquaculture practices has led to the emergence of several pathogens. Due to the indiscriminate use of antibiotics, multiresistant pathogenic bacteria have been isolated from aquaculture settings worldwide during the last decade [353]. For a sustainable further growth of this sector, novel strategies to control bacterial infections are needed. In this regard, preventative approaches are playing a greater role and will be increasingly more important in the future, as compared to reactive disease treatments [128].

Use of immunostimulants could be a promising approach for fish farmers in order to control disease losses in their facilities. Immunostimulants are in many cases PAMPs such as β-glucan or other compounds that activate the immune system through pattern recognition receptors such as TLRs and enhance overall resistance to various diseases [320]. Most studies assessing the immune effects of candidate molecules have focused on the activation of macrophages and granulocytes, i.e. the main immune cells of the innate system of fish. Stimulation of phagocytes with PAMPs such as β-glucan results in the respiratory or oxidative burst response, characterized by the production of reactive oxygen species with microbicidal activity. Stimulation also induces the production of cytokines that are essential factors for the functioning of innate and adaptive immune response [320]. For this kind of studies in fish, leukocytes derived from head kidney are widely used. This haematopoietic organ is rich in monocytes and macrophages that can be easily separated by density gradient centrifugation and cultured in vitro for several days [342]. Upon in vitro stimulation, primary HKL are able to implement important defence-related responses, including the production of ROS and pro-inflammatory cytokines [341,354].

An increasing number of AMPs have been isolated from fish and gained attention as potential immunostimulants in aquaculture [128]. In this study the immunomodulatory properties of a peptide, derived from the C-terminal region of the brown trout cathelicidin CATH1_SALTR, were investigated in comparison with the bovine cathelicidin BMAP-28. D'Este et al have recently characterized the effects of this latter peptide on the TLR4-mediated signalling in mouse macrophages, and found

that, besides its ability to completely neutralize the LPS molecules, this peptide was also able to selectively modulate the intracellular pathways triggered by TLR4-ligands [76].

In our hands, PMA and baker's yeast β–glucan elicited potent respiratory burst activity in freshly isolated primary HKL in a time- and dose-dependent manner (not shown), with sub-maximal responses at 0.5 μg/ml PMA and 125 μg/ml β-glucan, respectively. The different kinetics observed in each case reflect the specific molecular pathways triggered by each stimulus. Importantly, the combination of each AMP with PMA or β-glucan produced an increase in the respiratory burst, suggesting some effects of the AMPs under study on either cell membrane permeability or on the specific intracellular pathway involved. In this regard, the ability of the human cathelicidin LL-37 to increase the respiratory burst, alone or in combination with different stimuli in human and mouse phagocytes, has been reported by several studies [355-357], suggesting that this peptide may contribute to innate immunity by increasing the antimicrobial activity of phagocytes, but the underlying molecular mechanisms have not been clearly elucidated.

The kinetic behavior of the salmonid peptide STF was quite intriguing. This peptide was able to rapidly stimulate the production of ROS both, when added alone and when added in combination with PMA or β-glucan, with a faster kinetics compared to that elicited by PMA alone. A possible explanation could be a direct rapid interaction of the peptide with one or more components of the NADPH oxidase complex. In this regard, in the mid-90s Shi J. *et al*. demonstrated the interaction of the porcine cathelicidin PR-39 with the neutrophil cytosolic subunit p47phox with the consequence that in that case the assembly of the enzymatic complex was blocked and the respiratory burst inhibited [358]. An interaction with a membrane-localized subunit would appear more likely and, on the other hand, some interaction of the cationic peptide with the cell membrane can not be excluded. It is important to note, however, that the STF peptide, up to 32 μM, did not induce membrane permeabilization of HKL and RTG-2 cells, as assessed by LDH release assay.

When HKL were allowed to adhere overnight at 18°C and were subsequently exposed to PAMPs such as LPS or β-glucan for a more prolonged time period (1-8 h), an upregulated expression of pro-inflammatory cytokines (IL-1β, IL-8 and TNF-α) was observed, in line with the reported data [341]. None of the peptides under study was able to modulate the gene expression of the selected cytokines, at difference with the

atlantic salmon peptides asCATH1 and asCATH2 that have been recently reported to stimulate the transcription of IL-8 in peripheral blood leukocytes with a maximum effect at 8 h incubation [337]. This apparent discrepancy, however, could be explained by species-related specificities in host response, diversity in the cell population and different experimental settings.

Collectively, the results obtained underlie different properties of cathelicidin peptides derived from different animal species. Further in vitro studies on the effects of the selected AMPs on other important defence responses, such as e.g. phagocytosis or chemotaxis, could better clarify the immunomodulatory properties of the candidate molecules to be further investigated in in vivo studies.

6 CONCLUSIONS

Antibiotic resistance has become a major clinical problem in human and veterinary medicine [80]. The European Commission takes this problem so seriously that it has recently adopted a 5-year action plan [81] for the development of new antimicrobials, and the World Health Organization chose the theme "combact drug resistance" for the 2011 World Health Day. AMPs of the innate immune system are considered to be one promising class of leads on which to base novel antiinfective agents, owing to their broad-spectrum antimicrobial activity also towards multi-drug resistant microbial isolates and their scarce propensity to raise resistant mutants. In this regard, the present thesis was aimed to evaluate the functional and mechanistic properties of selected mammalian and fish AMPs in three different settings in view of their possible development as novel anti-infective agents for human and veterinary application.

The results obtained have pointed out the potential of BMAP-28 to be developed as antimicrobial agent owing to its capacity to inactivate both, *Prototheca* isolates from bovine mastitis and *Candida* isolates from female genital tract infections. Moreover, the therapeutic potential of BMAP-28 and LL-37 as coating material was also observed requiring further investigation towards this aim. Similar to BMAP-28, Bac5 and LAP were able to efficiently inactivate *Prototheca* isolates from bovine mastitis. Importantly, the ability of these peptides to act via novel, nonlytic mechanisms could be useful for the identification of druggable targets and development of selective therapeutic agents.

The selective enhancement of innate immunity by cationic AMPs represents a novel approach to (adjunctive) anti-infective therapy that complements directly microbicidal compounds [99]. In this regard, the results obtained in the third part of my thesis have highlighted the possible adjuvant activity of the salmonid cathelicidin STF owing to its ability to enhance important defence responses such as the respiratory burst when used in combination with a commercial immunostimulant.

Collectively, the results obtained highlight the antimicrobial and immunomodulatory properties of selected natural AMPs in three distinct settings, pointing out differences among peptides in potency, kinetics and mode of action, and

encourage further studies in view of the development of these molecules for human and veterinary applications

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8 ACKNOWLEDGMENTS

I am greateful to Dr. Renata Piccinini (Dipartimento Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Università degli studi di Milano) for providing us with the microbial isolates from bovine mastitis, and to Dr. Alessandra Arzese (Dipartimento di Scienze Mediche Sperimentali e Cliniche, Università degli Studi di Udine) for the isolation and characterization of *Candida* isolates from female genital tract infections. Moreover, Dr. Francesca Vita is gratefully acknowledged for technical assistance in SEM analysis (Dipartimento di Scienze della Vita, Università degli studi di Trieste). I also wish to thank Dr. Marco Scocchi (Dipartimento di Scienze della Vita, Università degli studi di Trieste) for providing the salmonid antimicrobial peptide STF. Prof. Marco Galeotti and Dr. Donatella Volpatti (Dipartimento di Scienze degli Alimenti, Università degli Studi Udine) are gratefully acknowledged for the supply of Rainbow trout fish and the support in Head kidney leukocytes separation. I am indebt to all members of Prof. Zanetti's lab, in particular to Dr. Francesca D'Este, Dr. Linda Tomasinsig and Debora Oro, for invaluable help. Finally I wish to express my sincere gratitude and appreciation to Dr. Barbara Skerlavaj and Prof. Margherita Zanetti for their guidance through this thesis.

This work was supported by Interuniversity Consortium for Biotechnology (CIB), Regione Friuli Venezia Giulia (Grant art. 23 L.R. 26/2005) and the Cross-Border Cooperation programme 2007-2013 INNOVAQUA.

9 ANNEX - SCIENTIFIC PUBLICATION

Research Article

Journal of PeptideScience

Received: 27 July 2011 Revised: 7 September 2011 Accepted: 8 September 2011 Published online in Wiley Online Library

(wileyonlinelibrary.com) DOI 10.1002/psc.1422

Comparative activity and mechanism of action of three types of bovine antimicrobial peptides against pathogenic *Prototheca* spp.

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The yeast-like algae of the genus *Prototheca* are ubiquitous saprophytes causing infections in immunocompromised patients and granulomatous mastitis in cattle. Few available therapies and the rapid spread of resistant strains worldwide support the need for novel drugs against protothecosis. Host defence antimicrobial peptides inactivate a wide array of pathogens and are a rich source of leads, with the advantage of being largely unaffected by microbial resistance mechanisms. Three structurally diverse bovine peptides [BMAP-28, Bac5 and lingual antimicrobial peptide (LAP)] have thus been tested for their capacity to inactivate *Prototheca* spp. In minimum inhibitory concentration (MIC) assays, they were all effective in the micromolar range against clinical mastitis isolates as well as a *Prototheca wickerhamii* reference strain. BMAP-28 sterilized *Prototheca* cultures within 30–60 min at its MIC, induced cell permeabilization with near 100% release of cellular adenosine triphosphate and resulted in extensive surface blebbing and release of intracellular material as observed by scanning electron microscopy. Bac5 and LAP inactivated *Prototheca* following 3–6 h incubation at fourfold their MIC and did not result in detectable surface damage despite 70–90% killing, suggesting they act via non-lytic mechanisms. In circular dichroism studies, the conformation of BMAP-28, but not that of Bac5 or LAP, was affected by interaction with liposomes mimicking algal membranes. Our results indicate that BMAP-28, Bac5 and LAP kill *Prototheca* with distinct potencies, killing kinetics, and modes of action and may be appropriate for protothecal mastitis treatment. In addition, the ability of Bac5 and LAP to act via non-lytic mechanisms may be exploited for the development of target-selective drugs. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cathelicidin; beta-defensin; antimicrobial peptide; *Prototheca*; antimicrobial activity

Introduction

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system. They have been widely detected in organisms ranging from bacteria and moulds to plants and animals [1]. A distinctive trait of these molecules is their capacity to exert direct antimicrobial effects *in vitro* on a wide array of bacterial and fungal pathogens. Many AMPs display cationic and amphipathic structures that favour interaction with biological membranes, so their antimicrobial mechanisms are often based on membrane disruption [2], whereas others use different mechanisms involving interference with vital membrane located processes [3] or internalization and interaction with intracellular components [4].

Among the various known families of animal AMPs, the well-characterized cathelicidins and defensin gene families seem to play a particularly important role in mammalian host defence. Members of both peptide families have been detected in phagocytic and epithelial cells [5,6], and in addition to protecting the host against invading microbes through their direct antimicrobial activity they also appear to act as signal molecules that variously modulate other components of the innate and adaptive immune responses [7,8] as well as to promote wound healing [9]. As they are produced at sites most in contact with the external environment, they may provide an important means of controlling the microbiota living within the animal and act as an effective first line of defence against opportunistic pathogens.

Unicellular achlorophyllous algae of the genus *Prototheca* have been identified as emerging agents of infection in humans and in other animals [10,11]. They are ubiquitous saprophytes and have been isolated from environmental reservoirs such as food, milk, soil, water and the faeces of domesticated animals [10]. *Prototheca* cells are ovoid to spherical in shape and range from 3 to 30 μm in diameter, depending on the species and stage of the life

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Abbreviations: *AMB, amphotericin B; AMP, antimicrobial peptide; CD, circular dichroism; CFU, colony forming unit; ESI-MS, electrospray ionization-mass spectrometry; LAP, lingual antimicrobial peptide; LUV, large unilamellar vesicle; MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PC/SM/Er, L-α-phosphatidylcholine/sphingomyelin/ergosterol; PG/dPG, phosphatidyl/diphosphatidyl-DL-glycerol; SEM, scanning electron microscopy; TFE, trifluoroethanol.*

cycle [10]. These algae reproduce asexually by multiple fission, giving rise to mother cells (sporangia) from which a variable number of endospores are passively released upon rupture of the sporangium.

The predominant form of protothecosis in cattle is mastitis, an inflammatory process of the mammary gland that is caused by multiplication of pathogens in its lumen and which generally requires some form of antibiotic treatment of infected herds. *Prototheca zopfii* genotype 2, in particular, causes acute to chronic granulomatous mastitis in cattle [12,13], with reduced milk production and atresia of the udder. Human forms of protothecosis are instead mainly caused by *Prototheca wickerhamii* and include cutaneous lesions, olecranon bursitis and systemic infections, particularly in immunocompromised patients [11]. *Prototheca* spp. are reported to be sensitive to amphotericin B (AMB) and variably susceptible to azoles and other drugs *in vitro* [10,11,14]. However, the *in vivo* efficacy of these drugs in mastitis treatment is unpredictable [10], and the only completely effective control measure to date is culling of infected animals [15].

The need for novel agents effective against *Prototheca* is highlighted by the increasing incidence of infection in dairy herds worldwide [13,16,17] and by the rapid spread of *Prototheca* isolates resistant to the few currently available antimicrobial drugs [16,18]. AMPs may be promising candidates for the development of such drugs as they rapidly kill a broad spectrum of microbes *in vitro*, also including bacterial mastitis pathogens [19,20]. The involvement of AMPs in udder infection is in any case suggested by the observation that some of them are produced by bovine mammary gland epithelial cells [21,22] as well as by activated neutrophils recruited during infection [19,23] and are secreted from these cells into bovine milk [24]. At least seven bovine cathelicidin peptides belonging to several distinct structural classes [25] and 26 different bovine defensins or the genes encoding them [26] have been reported. In the present study, two bovine cathelicidin peptides, bovine myeloid antimicrobial peptide (BMAP-28) and bactenecin 5 (Bac5), and the β-defensin lingual antimicrobial peptide (LAP) have been examined comparatively for their *Prototheca*-inactivating properties to provide functional and mechanistic information in view of their possible development as effective anti-*Prototheca* drugs. The three peptides were selected on the basis of distinct structural features, i.e., BMAP-28 is a linear, amphipathic α-helical peptide [27], Bac5 likely has an extended poly-L-proline type II conformation [28,29], and LAP has a β-sheet core stabilized by three disulfide bridges [26]. The effects of these peptides against several *Prototheca* isolates were investigated in terms of antimicrobial activity and related to their capacity to permeabilize the cell membrane and affect the cell morphology.

Materials and Methods

Peptide Synthesis and Characterization

Solid phase synthesis of BMAP-28 and Bac5 (Table 1), using Fmoc-chemistry, has been described previously [27,30]. The β-defensin LAP (Table 1) was synthesized with a microwave-assisted Liberty synthesizer (CEM Corp., Matthews, NC, USA) on Lys(Boc)-substituted Tentagel resin (substitution 0.16 meq/g, 0.05 mmol scale, Novabiochem, UK) essentially as described previously [31,32]. The good quality of the crude, fully reduced peptide (MW 4520.4) as verified by electrospray ionization-mass spectrometry (ESI-MS) (Esquire 4000, Bruker Daltonics Inc., Billerica, MA, USA) allowed oxidative folding without prior purification, as described previously [31]. Complete oxidation was confirmed by ESI-MS of the peptide (MW 4514.4), whereas the correct connectivity was partly established by ESI-MS analysis of the tryptic digest, in which the disulfide-linked fragments GICVPIR and QIGTCLGAQVK (MW 1870.9) confirmed the presence of the Cys^2–Cys^4 bridge. Peptide concentrations were determined in aqueous solution by measuring the absorbance at 257 or at 280 nm, taking into account the extinction coefficients of Trp and Tyr ($6839\ cm^{-1}\,M^{-1}$ at 280 nm) for BMAP-28 and Tyr ($1280\ cm^{-1}\,M^{-1}$ at 280 nm) for Bac5 [33,34]. The concentration of LAP was determined using the Waddell method [35].

Microbial Strains and Growth Conditions

All the isolates were collected from cases of clinical and subclinical mastitis in dairy herds from Lombardia region (Italy) and included 12 strains of *P. zopfii*, three strains of *Streptococcus agalactiae* and five strains each of *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus uberis*. The *Prototheca* isolates were identified as *P. zopfii* biotype II on the basis of biochemical features, essentially as described previously [14]. *P. wickerhamii* ATCC 16529 was the reference strain. The algae were grown on Sabouraud dextrose agar (Oxoid, UK) at 30 °C for 3–4 days, maintained in a refrigerator and transferred once a month to fresh medium. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. epidermidis* were maintained in Luria–Bertani agar plates (Oxoid), *S. uberis* and *S. agalactiae* in blood agar plates (Oxoid).

Antimicrobial Assays

Prototheca zopfii and *P. wickerhamii* were refreshed by streaking a loopful of algal cells onto Sabouraud dextrose agar. After incubation at 30 °C for 3 days, algae were harvested from the plates and resuspended in Sabouraud medium. The bacteria were cultured in Mueller–Hinton (Difco, Detroit, MI, USA) or brain heart infusion broth (Difco) (*S. uberis*) for 18 h, diluted 1:50 in fresh

Table 1. Sequences of bovine antimicrobial peptides

Peptide	Sequence	MW	q^a	%H[b]
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRI-NH_2	3074	+8	44
Bac5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFP-NH_2	5147	+10	30
LAP	GVRNSQS**C**RRNKGI**C**VPIR**C**PGSMRQIGT**C**LGAQVK**CC**RRK[c]	4514	+10	37

[a] *q*, charge.
[b] %H, percent of hydrophobic residues (A, V, M, L, I, F, Y, W). Cystine residues are considered to be hydrophobic.
[c] Cysteine residues involved in disulfide bridge formation are in bold. The cysteine arrangement is 1–5, 2–4, 3–6.

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medium and allowed to grow in a shaker at 37 °C. Mid-log-phase bacteria were harvested after 10 min of centrifugation at 1000 *g* and resuspended in appropriate media. The density of microbial suspensions was assessed by measuring the turbidity at 600 nm, with reference to previously determined standards, and adjusted to give 1×10^5 and 5×10^5 colony forming units (CFU)/ml for *Prototheca* and bacteria, respectively. The minimum inhibitory concentration (MIC) was determined by a broth microdilution assay in 96-well microtiter plates, essentially as described [34]. For *P. wickerhamii*, after the determination of the MIC, 50 µl aliquots of the assay mixture were plated on Sabouraud dextrose agar plates and incubated for 3 days at 30 °C in order to determine the minimum fungicidal concentration (MFC).

The microbicidal activity against *P. wickerhamii* ATCC 16529 was assayed by incubating cells (1×10^5 CFU/ml) at 30 °C in the presence of different concentrations of each peptide in Sabouraud. At fixed time points, culture samples were serially diluted in ice-cold physiological salt solution, and 50 µl aliquots were plated onto solid Sabouraud. After 3 days of incubation at 30 °C, colonies were counted and the CFU per millilitre calculated. The concentration of peptide that inhibited the growth of half of an inoculum of *P. wickerhamii* (IC_{50}) was estimated from dose–response curves, using the non-linear regression fit function of GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Cytotoxicity Assay

BME-UV1 cells were purchased from Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna and cultured at 37 °C with 5% CO_2 in the following medium: 40% Ham's F 12, 30% RPMI 1640, 20% NCTC 135, 10% foetal bovine serum containing 0.1% lactose, 0.1% lactalbumin hydrolysate, 1.2 mM glutathione, 10 µg/ml L-ascorbic acid, 1 µg/ml hydrocortisone, 1 µg/ml insulin, 5 µg/ml transferrin and 0.5 µg/ml progesterone. Cells were seeded in 96-well plates at a density of 2.5×10^5 cells/cm^2, grown for 24 h and incubated in complete medium in the absence or the presence of peptides. After a 24-h incubation, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma-Aldrich, St Louis, MO, USA) was used to assess cell viability according to the manufacturer's instructions.

ATP Bioluminescence Assay

Prototheca wickerhamii ATCC 16529 cells (1×10^5 CFU/ml) were incubated at 30 °C for 10–60 min with each peptide in Sabouraud medium. Cells were then centrifuged (1,000 *g*, 10 min) and the supernatants placed on ice until assayed for extracellular adenosine triphosphate (ATP). Cell pellets were submerged in liquid nitrogen for 5 min and resuspended in boiling water. Samples were then boiled for 5 min, centrifuged and the supernatants placed on ice until assayed for intracellular ATP. ATP levels were determined by luminometry using a luciferin–luciferase assay kit (Invitrogen, Carlsbad, CA, USA), and the amount of extracellular ATP was expressed as percentage of the total cellular ATP (extracellular + intracellular).

Preparation of Liposomes

Large unilamellar vesicles (LUVs) were prepared by extrusion of anionic phosphatidyl/diphosphatidyl-DL-glycerol [PG/dPG, 95:5 (w/w)] from egg yolk lecithin and bovine heart, respectively. Zwitterionic LUVs were prepared using neutral L-α-phosphatidylcholine and sphingomyelin from egg yolk and ergosterol dispersions [PC/SM/Er, 40:40:20 (*w/w*)]. All components were purchased from Sigma-Aldrich. Dry lipids were dissolved in chloroform, evaporated under a stream of nitrogen, and the residue was vacuum-dried for 3 h. The lipid cake was resuspended to a concentration of 3 mg/ml in the appropriate buffer by spinning the flask at a temperature above the lipid critical temperature. The resulting multilamellar vesicle suspensions were disrupted by several freeze–thaw cycles prior to extrusion with a miniextruder (Avanti Polar Lipids Inc., Alabaster, AL, USA) through polycarbonate filters with 100 nm pores. For all experiments, LUVs were freshly prepared or used within 1 or 2 days.

Circular Dichroism

Circular dichroism (CD) spectroscopy was performed on a J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA), using 2-mm quartz cells and 20 µM peptide with phospholipid vesicles in phosphate-buffered saline (PBS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.0) at room temperature (25 °C). Peptide/lipid suspensions (molar ratio 1:20) were incubated for 30 min at 37 °C before use. These spectra were compared with those obtained in the absence of LUVs (aqueous buffer), in the presence of trifluoroethanol (TFE), a solvent known to stabilize ordered conformations, and in the presence of anionic LUVs composed of PG/dPG that mimic bacterial membranes. Spectra were the average of at least two independent experiments, each with an accumulation of three scans.

Scanning Electron Microscopy

About 1×10^7 CFU/ml of *P. wickerhamii* ATCC 16529 were incubated at 30 °C in Sabouraud medium with each peptide. After incubation for 1 or 6 h at a peptide concentration (40 µM) that ensured 70–90% killing by all peptides, 15 µl of cell suspensions were deposited onto polylysine-coated glass coverslips and fixed at 4 °C overnight with 2.5% (*v/v*) glutaraldehyde in 0.2 M phosphate buffer, pH 7.3. The coverslips were then extensively washed with PBS, post-fixed at 4 °C for 1 h with 1% (*v/v*) osmium tetroxide in PBS and dehydrated in graded ethanol solutions. After lyophilization and gold coating, the samples were examined on a Leica Stereoscan 430i instrument (Leica Inc., Deerfield, IL, USA).

Statistical Analysis

Statistical differences among groups of data were analysed by one-way analysis of variance followed by the Bonferroni post test, using GraphPad Prism version 5.0. In all comparisons, $P < 0.05$ was considered significant.

Results

Antimicrobial and Permeabilizing Activity

The antimicrobial activity of BMAP-28, Bac5 and LAP (amino acid sequences reported in Table 1) was assayed against various microbial strains isolated from cases of bovine mastitis, including non-photosynthetic algae of the genus *Prototheca* and a number of contagious (*S. aureus* and *S. agalactiae*) and environmental (*S. uberis*, *S. epidermidis*, *E. coli* and *K. pneumoniae*) bacterial species.

In the standard microdilution assay, BMAP-28 was effective in the low micromolar range against all strains (Table 2), with the

Table 2. Antimicrobial activity of bovine peptides against isolates from cases of mastitis

Organism (no. of tested strains)	MIC range (μM)		
	BMAP-28	Bac5	LAP
Prototheca spp. (12)[a]	2–8	0.5–2	4–16
Staphylococcus aureus (5)[b]	2–4	>32	>32 (4)[c]
Streptococcus agalactiae (3)[b]	2	4–16	>32
Streptococcus uberis (5)[d]	2 to >32	16 to >32	>32
Staphylococcus epidermidis (5)[b]	1–2	1–2	32 (1)[c]
Escherichia coli (5)[b]	4–8	0.5–1	32 (8)[c]
Klebsiella pneumoniae (5)[b]	1–2	1–4	>32 (32)[c]

[a]Determined in Sabouraud broth.
[b]Determined in 100% Mueller–Hinton broth.
[c]Values in parentheses determined in 25% Mueller–Hinton broth.
[d]Determined in brain heart infusion broth.

exception of a single *S. uberis* isolate that was resistant to this peptide up to the highest concentration tested (32 μM). Bac5 was as effective as or even better than BMAP-28 against *Prototheca* spp. This AMP was also very effective against Gram-negative organisms while displaying high MIC values against most Gram-positive organisms, particularly *S. aureus* and *S. uberis*, in line with the reported selectivity of Pro-rich AMPs for Gram-negative species [4]. Under the same assay conditions, the β-defensin LAP inhibited the growth of *Prototheca* spp. with an average MIC value of 8 μM but was ineffective against all bacterial isolates even at the highest concentration tested (32 μM) (Table 2). LAP did however display a significant antibacterial activity when assayed in fourfold diluted bacterial growth media (Table 2, values in parentheses). These results are in agreement with the widely reported salt and medium sensitivity of the antibacterial activity of β-defensins [36], whereas the anti-algal activity appears to be more robust.

The MIC data reported in Figure 1 indicate that all three peptides are effective against all *Prototheca* isolates. An increasing number of studies indicate that this algal microorganism is an emergent mastitis pathogen refractory to antibiotic therapy [18], and particularly, *P. wickerhamii* has been shown to affect humans by producing cutaneous infections in immunocompromised patients [37]. These considerations prompted us to further investigate the antimicrobial effects of BMAP-28, Bac5 and LAP against *P. wickerhamii* ATCC 16529 as a reference strain. Its

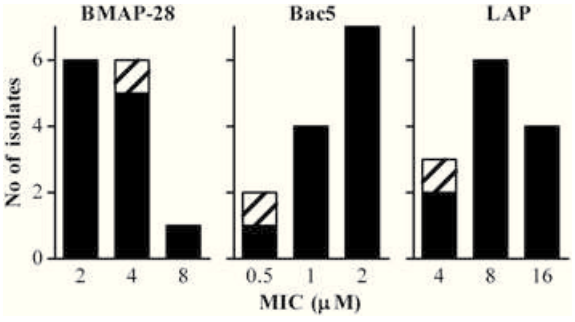

Figure 1. Distribution of MIC values of the indicated peptides for *Prototheca* spp. isolates. The hatched part of the bars refers to *P. wickerhamii* ATCC 16529.

growth was inhibited by the AMPs with MIC values ranging from 0.5 μM (Bac5) to 4 μM (BMAP-28 and LAP), as compared with a MIC of 0.2 μM for AMB (Table 3), which has been already reported to be active against this strain [11]. In a microbicidal activity assay, BMAP-28 was found to decrease the viability of *P. wickerhamii* by approximately 80% in only 10 min (Figure 2A) and by >95% within 60 min at a peptide concentration corresponding to its MIC value (4 μM) (Figure 2A and B). The microbicidal effects of LAP and Bac5 were instead significantly slower. At their MIC concentration, they were not effective against *P. wickerhamii* up to 30 min and caused an approximately 20% decrease in cell viability within 60 min (Figure 2A). Accordingly, the IC_{50} calculated following 10 and 60 min incubation was considerably higher for Bac5 and LAP than for BMAP-28 (Table 3). Increasing the concentration of Bac5 and LAP to

Table 3. Antimicrobial activity against *P. wickerhamii* ATCC 16529

Test agent	MIC (μM)	MFC (μM)	IC_{50} (μM)	
			10 min	60 min
BMAP-28	4	4	2	1
Bac5	0.5	2	6	2
LAP	4	16	32	16
AMB	0.2	>1.6	—	—

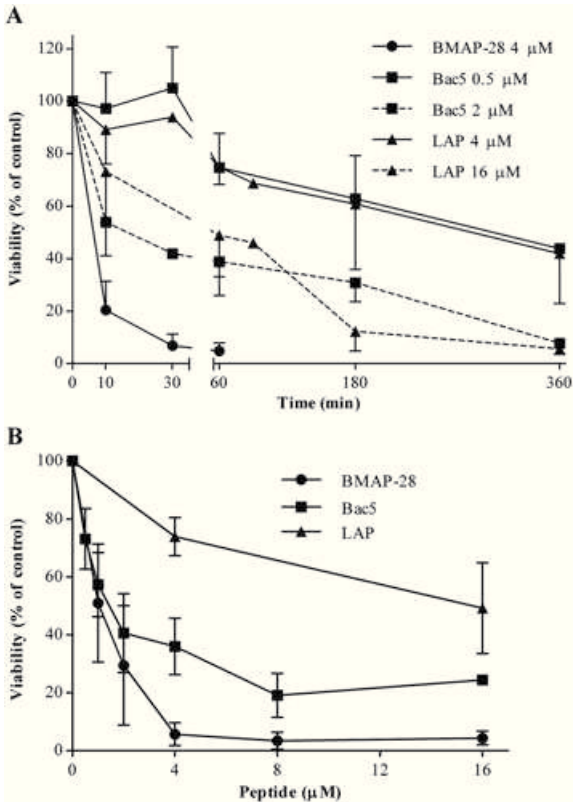

Figure 2. Time-killing curve (A) and dose–response after 60 min incubation (B) of BMAP-28, Bac5 and LAP against *P. wickerhamii* ATCC 16529. Cells were incubated with peptides at the indicated concentrations in Sabouraud medium. At selected time points, samples were serially diluted and plated to allow colony counts. The results are reported as percent viability relative to untreated cells and are the mean ± SD of at least three independent experiments.

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16 μM resulted in 60% and 40% killing activity, respectively, following 60 min cell incubation (Figure 2B), whereas complete killing was only achieved following 6 h incubation of algal cells with each peptide at its MFC value, corresponding in both cases to four times the MIC value (i.e., 2 μM Bac5 and 16 μM LAP) (Figure 2A). Importantly, none of the peptides was cytotoxic for BME-UV1 cells, used as a model for bovine mammary gland epithelium [38], at its algicidal concentration (cell viability higher than 95% as assessed by MTT assay). The viability of these cells was only slightly affected by BMAP-28 (approximately 80% viable cells) at fourfold its algicidal concentration, whereas Bac5 and LAP were ineffective at corresponding concentrations. Overall, these data indicate a much lower affinity of these peptides for BME-UV1 as compared with *Prototheca* cells. This is in line with the results of numerous studies indicating that AMPs preferentially associate with target microorganisms rather than with host cells [2,7]. Intrinsic molecular and structural characteristics of microbial versus host cells as well as inherent structural features of AMPs are thought to be major determinants of this selective antimicrobial discrimination [2].

To gain further insight into the mechanism of antimicrobial action, peptides were analysed for their capacity to induce extracellular release of ATP from *P. wickerhamii* as a means to evaluate cell membrane perturbation. At its MIC value, BMAP-28 induced, respectively, 80% and 100% release of cellular ATP after 10 and 60 min exposure (Figure 3), consistent with a rapid membranolytic activity. In contrast, Bac5 and LAP did not promote ATP release for up to 60 min incubation at their MIC values. This was confirmed by a lack of propidium iodide uptake under the same experimental conditions (not shown). Approximately 50% ATP release was observed after cell incubation with Bac5 at fourfold MIC value. Under the same concentration conditions, LAP caused approximately 25% permeabilization (Figure 3).

CD Spectroscopy

A different propensity of the peptides for interaction with the plasma membrane was also suggested by CD spectra determined in the presence of zwitterionic LUVs containing ergosterol, a simple model for algal cell membranes. As shown in Figure 4, the CD spectrum of BMAP-28 showed a strong conformational transition from random coil in aqueous buffer to α-helix in the presence of 50% TFE (Figure 4A). Under these conditions, all BMAP-28 molecules likely have a similar helix content, which was estimated at approximately 50% [39], compatible with formation of an N-terminal helix and disordered C-terminal tail [27]. The spectrum in the presence of zwitterionic LUVs indicates a lower yet detectable helix content, which may be explained by a smaller part of the peptide being helically structured or, more likely, only part of the peptide population being membrane bound in a helical conformation, estimated at about 20% by comparison with the TFE spectrum. The CD spectrum of BMAP-28 in the presence of anionic vesicles was considerably more intense, and the minimum shifted from about 208 to 222 nm. This indicates a stronger membrane interaction and is possibly consistent with helix aggregation at the membrane (as suggested by the altered $\theta^{208}/\theta^{222}$ ratio [40]) and/or increased content of β-structure, presumably at the C-terminus of the sequence (Figure 4A).

Bac5 showed similar spectra in aqueous buffer and in the presence of neutral LUVs (Figure 4B), indicating that its conformation was not markedly altered in their presence. The spectra are consistent with an extended type II poly-L-proline conformation, in accordance with previous reports [28,29]. Similar slight changes in the shape of the CD spectrum were observed in the presence of TFE or anionic LUVs. Taken together, these results indicate that Bac5 does not markedly alter its conformation in the presence of biological membranes and are more consistent with a surface interaction (especially with anionic LUVs where it is favoured by electrostatic attraction) than membrane insertion as for BMAP-28.

The CD spectrum profile of LAP measured in aqueous solution was consistent with a partly β-sheet, partly random coil conformation (Figure 4C) and was unaffected in the presence of zwitterionic LUVs, indicating poor interaction of the peptide with this type of membrane. A helical component became evident in the presence of TFE or of anionic vesicles (Figure 4C), likely due to structuring of the N-terminal segment, as also observed for hBD3 under similar conditions, and may result from some form of interaction with bacterial-type membranes [41].

SEM Analysis of Surface Alterations

To further investigate the mechanism of action, we used scanning electron microscopy (SEM) to visualize morphological alterations induced by each peptide, which could be related to its killing mechanism(s). Representative micrographs of untreated *P. wickerhamii* showed individual cells with ridged surface (Figure 5A) or morula-like structures (Figure 5B) typical of *P. wickerhamii* sporangia. As shown in Figure 5C and D, incubation for 60 min with BMAP-28 resulted in extensive surface blebbing on both individual and sporangial cells, with occasional release of intracellular material (Figure 5C). These features support a membranolytic killing mechanism. LAP-treated cells did not reveal evident signs of surface damage, although we consistently found that approximately 15% of treated cells had an uncharacteristic

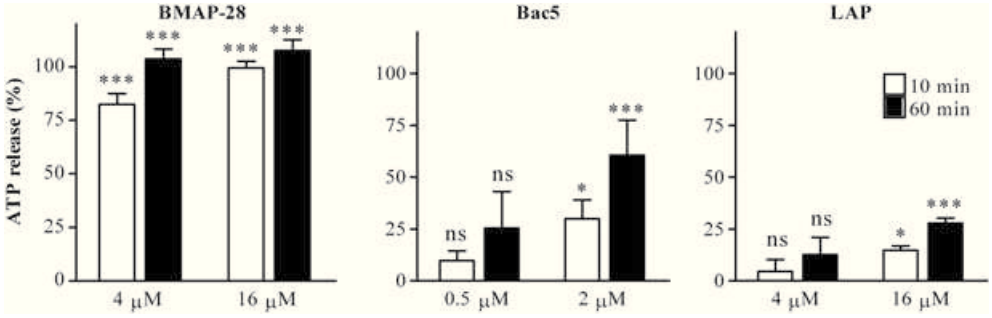

Figure 3. ATP release from peptide-treated *Prototheca* cells. Cells were incubated for 10 and 60 min with the indicated peptides at MIC and fourfold MIC values. The amount of ATP in the cell supernatants was expressed as percentage of the total cellular ATP. Results are the mean ± SD of at least three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

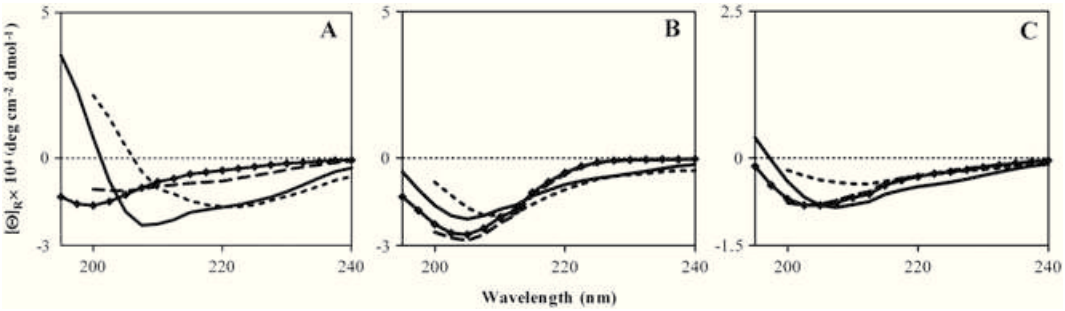

Figure 4. CD spectra of BMAP-28 (A), Bac5 (B) and LAP (C) in PBS (diamonds), 50% TFE (solid line), PG/dPG (short dashes) and PC/SM/Er (long dashes) LUVs. The concentrations of peptide and lipid in PBS were 20 and 400 μM, respectively.

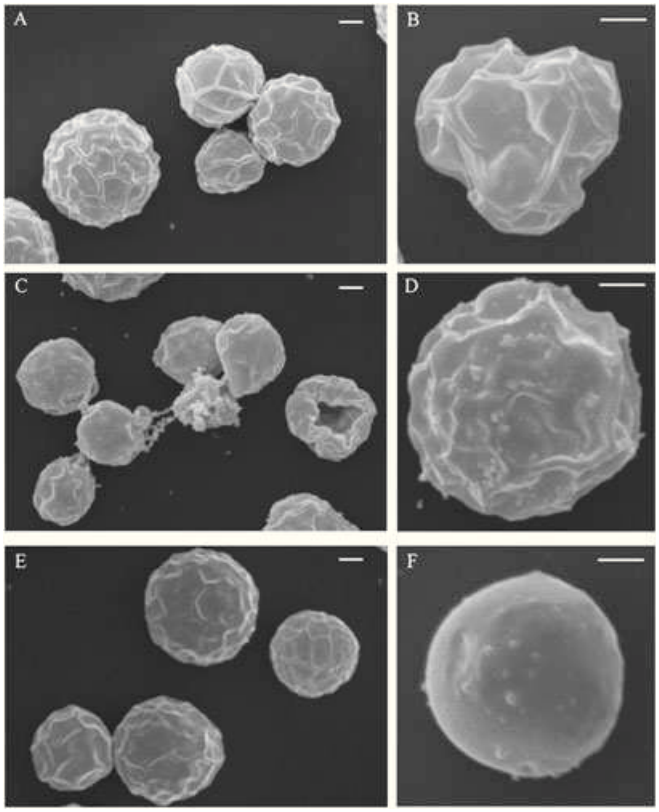

Figure 5. Scanning electron microscopy of *P. wickerhamii* cells incubated for 60 min in the absence (A, B) or in the presence of 40 μM BMAP-28 (C, D) or 40 μM LAP (E, F). Magnification, ×15 000; bars, 1 μm.

smooth, unridged surface (also showing some surface blebbing), as compared with only about 2% of untreated control cells showing this feature (Figure 5E and F). Cell incubation with Bac5 did not produce any visible surface alterations, even after a 6-h incubation time (not shown).

Discussion

The efficacy of currently available pharmacological therapies for the treatment of *Prototheca* infection is controversial [11,16] and is in any case undermined by the emergence of resistant strains worldwide to the few drugs in use [18]. Naturally occurring AMPs may be a promising source for the development of effective anti-prototheca agents, as these peptides generally show a rapid and broad-spectrum antimicrobial activity and are largely unaffected by resistance mechanisms to clinically used antiinfective agents [42,43]. In this regard, it has been reported that peptides derived from bovine lactoferrin, a multifunctional protein endowed with antimicrobial activity [44], show the ability to inactivate *P. zopfii* with MIC value of 1 μg/ml, although the mechanism underlying this activity has not yet been elucidated [45]. In this study, we have examined the anti-prototheca activity of chemically synthesized versions of the endogenous bovine AMPs BMAP-28, Bac5 and LAP. These peptides are highly cationic (+8 to +10), with one out of every three/four residues being positively charged, and have a variable content of hydrophobic amino acids, ranging from 30% in Bac5 to almost 50% in BMAP-28. The latter is a linear peptide with a propensity for adopting an α-helical conformation in the region encompassing residues 1–18, in a membrane-like environment [27]. The alternation of hydrophobic and hydrophilic residues confers a strong amphipathic

character to the helix, favouring interaction with biological membranes [27]. Bac5 includes in its sequence a tandemly repeated Xaa1-Pro-Pro-Xaa2 motif, in which Xaa1 is almost invariably Arg and Xaa2 is a bulky hydrophobic residue (Ile, Phe or Leu) [46]. On the basis of CD and nuclear magnetic resonance spectroscopy, an extended poly-L-proline type II conformation has been proposed [28,29], but this or other possible conformations do not result in amphipathic structures, judging from Edmundson-type projections (www.bbcm.units.it/~tossi/HydroMcalc). LAP has the same global positive charge as Bac5 and an intermediate content of hydrophobic residues between BMAP-28 and Bac5. Mapping its residues on the known structures of the human defensins hBD2 and hBD3 and bovine defensin 12 (PDB ID: 1FD3, 1KJ6 and 1BNB respectively) indicates that it could have a roughly discoid structure which is predominantly polar, with a small hydrophobic patch on one edge.

Antimicrobial assays indicate that all peptides are effective *in vitro* against mastitic isolates of *P. zopfii* and kill a reference strain of *P. wickerhamii* in a manner dependent on time and dose, at concentrations comparable with or slightly higher than that of AMB. The observed decrease in CFU following treatment with BMAP-28 at its MIC value was quantitatively and kinetically correlated with the extent of ATP released from *P. wickerhamii*, suggesting a mainly membranolytic mode of action. A killing mechanism based on membrane perturbation is considered the norm for α-helical AMPs and underlies a broad-spectrum activity against Gram-positive and Gram-negative bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* clinical isolates [27,34], as well as the fungi *Cryptococcus neoformans* and several *Candida* spp. [47].

The proposed lytic mechanism for BMAP-28 is supported by CD spectra which indicate capacity to interact with anionic LUVs (models for bacterial membranes) and with ergosterol-containing zwitterionic liposomes (models for fungal/algal membranes). The plasma membrane of *Prototheca* is rich in zwitterionic phospholipids (>70% by weight of total phospholipids) and ergosterol (4% of total neutral lipid *w*/*w*) [48]. Our results are consistent with published evidence that BMAP-28 induces substantial calcein release from artificial liposomes with a composition mimicking that of fungal membranes and causes rapid uptake of the dye SYTOX green into the hyphae of the plant pathogen *Fusarium oxysporum* f. sp. *vasinfectum* [49]. Overall, these results point to the plasma membrane as a primary target for the antifungal and anti-algal activity of BMAP-28.

Bac5 and LAP exhibited a different killing behaviour. Whereas BMAP-28 sterilized *P. wickerhamii* cell cultures within 30 min incubation at its MIC, Bac5 and LAP led to a comparable decrease in CFU only after 3–6 h incubation, and only at their MFC, which corresponded to four times the MIC for both peptides. This, and the fact that, unlike BMAP-28, only modest amounts of ATP were released from *Prototheca* cells following up to 1 h incubation with Bac5 or LAP at MIC values, argue against a lytic killing mechanism. The amount of ATP in the supernatants of cells treated with lethal concentrations of these peptides increased at longer exposure times but was not proportional to the CFU reduction measured in parallel, so likely resulted from leakage of cell contents as a late effect of killing rather than from early membrane injury. This is also backed by CD studies, which suggest a looser interaction of Bac5 and LAP with neutral model membranes. The structure of Bac5 is also unaffected in the presence of phosphatidyl choline vesicles [28], and no calcein release was observed from this type of vesicle in the presence of synthetic fragments of Bac5 and its porcine analogue PR-39 [29,50,51]. The CD behaviour of LAP in the presence of neutral or anionic model membranes is similar to that of hBD3, whose weak propensity to interact with neutral liposomes has also been demonstrated using high-resolution synchrotron small-angle X-ray scattering [52]. In short, the conformation of either peptide is little affected by the presence of neutral membranes, whose integrity is likewise little affected by peptide interaction. Significantly, human β-defensins have been shown to kill fungal cells without causing membrane disruption [53].

The differential killing mechanisms of the three peptides, and the fact that only BMAP-28 acts via membrane permeabilization is further supported by SEM analysis of peptide-treated cells, revealing extended surface damage only following incubation with BMAP-28, even at Bac5 and LAP concentrations that caused 70–90% killing and after prolonged incubation times. In this respect, members of both the Pro-rich and β-defensin AMP families have been variously shown to penetrate into bacterial or mammalian cells without compromising plasma membranes [30,31,54,55]. An accepted mechanistic model for the killing of Gram-negative bacteria by Pro-rich peptides involves interaction with intracellular targets following cellular internalization mediated by specific membrane transporters [4]. With respect to the antifungal activity of β-defensins, it appears that different members may act via distinct mechanisms, dependent on interaction with different cell wall components, which in turn are different from those used by neutrophil defensins or plant defensins [56], indicating they act in a quite complex framework.

To our knowledge, this is the first report on the activity of AMPs against *Prototheca* spp apart from lactoferrin. We demonstrate that BMAP-28, Bac5 and LAP kill this organism with distinct potencies, killing kinetics and mode of action and may be appropriate for treatment of protothecal mastitis. Additionally, the ability of Bac5 and LAP to act via novel, non-lytic mechanisms could be useful for the identification of druggable targets and development of selective therapeutic agents. Future studies are thus warranted to better elucidate the respective mechanisms of action and allow the design of AMPs or derivatives with increased potency.

Acknowledgements

This work was supported by the Italian Ministry of Education, University and Research (Progetti di Ricerca di Interesse Nazionale 2007), Interuniversity Consortium for Biotechnology (CIB) and Regione Friuli Venezia Giulia (Grant art. 23 L.R. 26/2005).

Francesca Vita is gratefully acknowledged for technical assistance in SEM analysis.

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