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ZI Buhroo
Temperate Sericulture Research Institute, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India

MA Bhat
Department of Studies in Sericulture Science, University of Mysore, Mysuru, Karnataka, India

GK Bali
Department of Zoology, Savitribai phule Pune University, Pune, Maharashtra, India

AS Kamili
Temperate Sericulture Research Institute, Sher-e- Kashmir University of Agricultural Sciences and Technology of Kashmir, Srinagar, Jammu and Kashmir, India

NA Ganai
Directorate of Planing and Monitoring, Sher-e-Kashmir University of Agricultural Science and Technology of Kashmir, Shalimar, Srinagar, Jammu and Kashmir, India

Correspondence
MA Bhat
Department of Studies in Sericulture Science, University of Mysore, Mysuru, Karnataka, India

Antimicrobial peptides from insects with special reference to silkworm *Bombyx mori* L: A review

ZI Buhroo, MA Bhat, GK Bali, AS Kamili and NA Ganai

Abstract
A characteristic of insect immunity is the rapid and transient activation of immune genes to produce effectors in response to microbial infections. The production and release of antimicrobial peptides (AMPs) are thought to play a major role in the innate immunity against invading pathogens. The antimicrobial peptide genes are induced by microbial challenge in the fat body (equivalent of the mammalian liver), followed by the secretion of these peptides into the haemolymph, which is the hallmark of the humoral reactions. These antimicrobial peptides from several families reach high concentrations in the haemolymph and efficiently kill invading microorganisms. Antimicrobial peptides (AMPs) are low molecular weight biologically active molecules having exciting properties. A wide variety of AMPs have been isolated from various sources like plants, animals, mammals, insects and microorganisms. The most familiar structure is represented by α-helical conformation in organic solutions or disulfide stabilized β-sheet with or without α-helical domains present. Despite of striking diversity in structure and chemical nature, all of them possess antimicrobial activity. This fundamental property makes them as a promising candidate compared to chemical antibiotics. In the near future attempts should be made to generate AMPs with higher antimicrobial activity and broad range of microbe action. In this review, focus is given on the AMPs found in the silkworm *Bombyx mori*, with some findings on the innate immunity responses (both cellular and humoral). This review also summarizes six classes of antimicrobial peptides (cecropin, defensin, attacin, leboncoin, moricin and gloverin) found in *B. mori* and their mode of actions against diverse range of pathogenic microbes.

Keywords: Antimicrobial, *Bombyx mori*, insects, silkworm

1. Introduction
Insects possess a very potent innate immune system to eliminate invading pathogens, which basically comprises cellular and humoral defense mechanisms against microbes. Pathogen recognition receptors of insects can directly bind to microbes and destroy them by encapsulation followed by phagocytosis. Alternatively, upon binding they can induce downstream signaling factors, which in turn provoke production of antimicrobial proteins, nodule formation, and melanization in specific tissues such as the fat body and haemocytes. *Bombyx mori* L. silkworms (commonly known as Mulberry Silkworm) are one of the major domesticated silkworms for producing commercial silk. The silkworm feeds on the Mulberry plant, which have different species under family Moraceae. Besides the production of good quality silk of commercial interest, the silkworm has been exploited for its different bioactive properties. Recently, there have been many studies carried out on possible antimicrobial peptides from *B. mori* L, wherein the major focus is on peptides of antimicrobial origin that exhibit different mechanism of action and lesser side effects. Many of enormous antimicrobial peptides (AMPs) have been produced from insects, mammals, reptiles and plants to protect against microbial infections and environmental changes. Several AMPs such as, cecropin, moricin, gloverins, attacin, ebonics and lebocin, which have a broad spectrum of antimicrobial activities, have been identified from the silkworm, *B. mori*. It is generally noticed that temperate bivoltine breeds are more susceptible to pathogens than tropical multivoltine breeds. [1].

2. What Are Antimicrobial Peptides?
Antimicrobial peptides are small molecular weight proteins having broad range of activity against bacteria, fungi and viruses. These biologically active peptides are synthesized by vast number of organisms as an essential factor for innate immune response. These peptides are considered as probable candidate for forthcoming drugs, because of their broad range of

activity, lesser toxicity and decreased resistance development by target cells. The smaller size of AMPs helps in the rapid diffusion and secretion outside the cells, which is mandatory for evoking immediate response against pathogenic microbes. Other necessary factors such as size, charge, hydrophobicity, amphi phatic stereo geometry and self-association with the biological membrane are critically essential for their broad spectrum antimicrobial activities [2]. Out of the knowledge gained from the past suggests that discovery of antimicrobial peptides makes natural antibiotics a key element for the generation of novel drugs for the treatment of bacterial and fungal infections [3-6] Moreover, the wide spectrum of antimicrobial activity of these peptides makes them potentially suitable in the treatment of cancer [7] and viral [8-10] or parasitic infections [11]. Lipid composition variation between prokaryotic and eukaryotic membranes is the main targets for AMPs. The antibacterial activity of some peptides lies in their ability to interfere with a specific mechanism of the microbial cell without affecting similar mechanisms present in the cells of the infected organism. A huge number of AMPs reported in the literature are already listed in the publicly available databases including Swissprot and TrEMBL, AMSDd, APD and ANTIMIC. The Antimicrobial Peptide Database (APD) offers a network to conclude the antimicrobial activity of any submitted sequence, based on a simple residue analysis and count method and some favourable statistical information on peptide sequence, function and structure. AMPs are commonly designated as peptides having less than 100 amino acid residues with an overall positive charge (usually +2 to +9). They have presence of multiple lysine and arginine residues and a substantial portion (≥30% or more) of hydrophobic residues [12]. They are mostly cationic in nature but few of them are anionic. Cationic peptides share the common property to fold into amphi phatic membranes, which is usually induced upon interaction with membrane or membrane mimics. Besides having antimicrobial properties against Gram-positive and Gram-negative bacteria, they are also effective towards fungi [13] and protozoa [14] with micromolar or submicromolar minimal inhibitory concentrations (MIC) [15].

3. Why Antimicrobial Peptides?

The rate of spread of multi resistant microbial strains is becoming a source of lethal infections. The antimicrobial drug mutations of bacteria, making them resistant to treatments are formerly effective. Antimicrobial drug resistance arises due to high rate of genetic mutation in microbes that make them resistant to formerly used antimicrobial agents which were found to be effective earlier. The property of bacterial strains for rapid transfer of their genes suggests that bacterial resistance to antibiotics occurs quickly in the evolution of bacterial development [16]. According to antimicrobial resistance report by World Health Organization (2014), common type of drug resistant bacteria includes *Escherichia coli*, *Klebsiella pneumonia*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Streptococcus pneumonia*, *Shigella* species that are acquired resistant against common antibiotics like cephalosporins, fluoroquinolones, carbapenems, methicillin, penicillin, including resistance conferred to extended spectrum beta lactamases stated that this serious threat is no longer a prediction for the future; it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country. The rise of fungal infections at the same time in the decades is due to increase of immune-compromised patients, including HIV/AIDS patients, oncology patients with chemotherapy induced neutropenia and transplant recipients who are receiving immunosuppressive therapy [17]. To combat with the present multi drug resistance predicament, the use of AMPs from insects becoming a major area of interest for the discovery of new antibiotics which is biologically active, less toxic and reduction of resistance. Insect AMPs become a major area of interest for the discovery of new antibiotics which are biologically active and less toxic to combat with the present multi drug resistance predicament.

4. Antimicrobial Peptides from Silkworm *B. mori* L.

Cellular defense response involves direct interactions between circulating hemocytes and microbes through phagocytes, nodulation and encapsulation [82, 83]. Humoral reactions involve inducted synthesis of antibacterial proteins [84]. In insects, a large number of antibacterial proteins have been isolated and these antibacterial proteins are classified in five major groups [85]. Cecropins, lysozyme and prolin-rich antibacterial proteins have been reported from immunized haemolymph of silkworm, *B. mori* [86, 87]. The detergent properties of these antimicrobial proteins disrupt the cell membranes of the invading microbes and enzymatically attack bacteria by hydrolyzing their peptidoglycan cell walls [88, 89]. Antimicrobial proteins play an important role in eliminating invaders. Antimicrobial proteins are amphiphilic, positively charged molecules that protect the host from infection [88].

4.1 Cecropins

Cecropins are cationic antimicrobial peptides, first isolated from the immunized haemolymph of the giant silk moth, *Hyalophora cecropia* [18, 19]. In insects three principles cecropins are present, *viz.* A, B and D having a length of 35 to 37residues which lacks cysteine with a strong basic N-terminal linked to a neutral C-terminal by a flexible glycine proline link [20]. Cecropin was also recognized as a part of immune response in two silkworm *B. mori* and *Antherea peryni*. Many families of cecropin have been isolated in lepidopteran and dipteran insects. Members of cecropins family include, sarcotoxin-I [21], papiliocin [22], stomoxyn [23], hinnavin [24], SB-37 and Shiva (synthetic derivatives of cecropins) [25]. Cecropins have broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria and also to fungi. The antimicrobial property of cecropin is governed by the amidation at the C-terminus and amidation is necessary for the interaction of cecropin with liposomes [26]. Besides antimicrobial property cecropin and cecropin derivatives (SB-37 and Shiva) are active against parasites like *Plasmodium* and *Trypanosoma* [27-29] and can inhibit the replication of HIV-1 virus [30] and proliferation of cancerous cells [31]. The antibacterial peptide gene of cecropin XJ was cloned in Pyes2/CT/a expression vector and expressed in *Saccharomyces cerevisiae* INCScI strain. This recombinant protein had shown strong antimicrobial activities against Gram-positive and Gram-negative bacteria. This study also suggests that yeast can be used system for the large production of this protein by genetic engineering methods [32]. A promoter from *B. mori* cecropin A1 (Cec A1) was cloned and characterized to study the transcriptional control of the antimicrobial peptide during immune challenges. From the deletion and mutation constructs it was affirmed that regulatory region is kB motif which increases the activity of

the promoter [33]. Cecropins maintain a random coil structure in aqueous solution but transformed to an alpha-helical structure in hydrophobic environments. *Hyalophora cecropia* cecropin. A having 37 residues contains two helical regions in residues 5-21 and 24-37 [34] and sarcotoxin IA (39 residues) display an N-terminal amphiphatic alpha-helix (residues 3-23) and a more hydrophobic C-terminal alpha helix (residues 28-38) joined by a hinge region (residues 24-27) [35].

4.2 Defensins

Defensins are cationic antimicrobial peptides (4 kDa) together with six conserved cysteine residues that form three intra-molecular disulfide bridges. These peptide are mainly subdivided based on their spacing pattern of the cysteines into five groups, particularly invertebrate, plant, α, β and θ subfamilies [36]. Insect defensin was first detected in flesh fly, *Sarcophoga peregrine* which contain six cysteine residues as *sapecins* [37] and in the immunized larvae of *Phormia terranova's* cationic peptides [38]. It includes phormicins, sapecins [39, 40] royalisin [41] and spodoptericin [42]. In contrast to antibacterial defensin only few antifungal defensins have been reported, drosomycin from *Drosophila*; heliomycin from the tobacco budworm *Heliothis virescens* [43], gallerimycin from greater wax moth larvae *Galleria mellonella* [44], termicin from the isopteran *P. spiniger* [45] and Alo13 from the harlequin beetle *Acrocinus longimanus* (Coleoptera) [46]. A gene related to defensin called Bm Defensin A was discovered in the silkworm genome. Reports suggest that 5'-upstream regulatory region of gene encoding Bm Defensin A contains cis elements such as NF-kβ binding site, an IL-6 responsive element and the GATA motif. Bm DefA open reading frame encodes a propeptide which consist a 22 residue signal peptide, a 34- residue propeptide and a 36-residue mature peptide having a molecular weight of 4 kDa. The presence of six cysteine motif in the mature peptide suggests a characteristics feature of insect defensin and isoelectric point was found out to be 4.12 which suggest that it is a novel anionic defensin. This is highly transcribed in the hemocyte, silk gland, head, and ovary of the silkworm larvae and in the fat body of early stage pupae and moth. BmDefA is strongly induced by immune challenge which represents its important role in both immunity and metamorphosis [47]. Bm Defensin B a homolog of defensin was indentified in the silkworm *B. mori.* The presence of six cysteine residues in conserved sequence is a crucial characteristic of all defensins. Bm Defensin B has low amino acid similarity (about 27%) with that of Bm Defensin A. The Bm Defensin B gene was expressed in the fat body and it was found that it is strongly activated by bacteria such as *Escherichia coli* and *Bacillus subtilis*, and also by *Beauveria bassiana*. On the other hand, the Bm Defensin A gene was expressed at lesser extent. The expression of Bm Defensin B gene was strongly stimulated by the Rel protein RelB and Relish. The results of the study suggested that Bm Defensin B gene expression is controled through both the Toll and Imd pathway. This strongly suggests that Bm Defensin B plays a crucial role in regulating immune reaction against both bacteria and fungi in *B. mori.* This study also demonstrates that Bm Defensin B is the first antimicrobial peptide gene activated by *B. bassiana* [48]. *Bombyx mori* defensin like peptide (BmDp) was identified by wdS20658 cDNA from the *Bombyx mori* Expressed sequence Tags (EST) database constructed from non-immune challenged cells in *B. mori*. This peptide was deposited in Genbank 2005, having Genbank accession number DQ118523. It was found that wdS20658 cDNA has consists of six cysteines [C…CXXXC…C…CXC] and showed high identity of 75% with spodoptericin. Semiquantitative RT-PCR analysis showed that gene expression of BmDp was inducible by bacterial injection immunization and it was highest after 8h of immunization. This finding suggested that BmDp is associated with immune response against bacteria. A purified form of the recombinant GSTmDp fusion protein when assayed did not show any significant activity against bacteria and fungi [49]. When mode of action of defensin was tested with the help of recombinant insect defensin using *Micrococcus luteus* as test organism it was reported that defensin disrupts the permeability barrier of cytoplasmic membrane of *M. luteus* which results in the lossof cytoplasmic potassium, partial depolarization of the inner membrane, decrease in cytoplasmic ATP and inhibits respiration. It was observed that permeability changes in the membrane reflect the formation of channels in the cytoplasmic membrane by defensin oligomers. These results were strongly supported by patch-clamp experiments that show that insect defensins form channels in giant liposome [50].

4.3 Moricin

Moricin was isolated from the haemolymph of *Bombyx mori* which was found out to be active against *Staphylococcus aureus* [51]. The basic nature of moricin may be important for the attachment of positively charged peptides to the negatively charged bacterial surfaces through electrostatic interaction [52]. Moreover, the presence of amphiphatic α-helix is mainly responsible for antibacterial activity. There is a presence of charged amino acids at intervals of three of four amino acid residues in the N-terminal half of moricin, implying a distinctive structure in antibacterial proteins containing the amphiphatic which shows a characteristic α-helix [53]. Although, the main target for moricin is bacteria but it shows slight antifungal activity against some strains of yeast. Despite the fact that both morrisons and cecropins act against several species of bacteria, moricin tends to have higher activity against Gram-positive bacteria. Bacterial membranes are mainly targeted by moricin and the C-terminal fragment is mainly responsible for attaching with the membrane by which it changes the permeability of the membrane by N –terminal amphiphatic a-helix. Further, the absence of modifications in the moricin such as α-amidation of C termini in the hydroxylation of Lys residues in *B. mori* cecropins, the O-glycosylation of Thr residues in proline-rich antibacterial peptides or formation of intermolecular disulfide bonds in defensins gives a exclusive property to moricin for the production of the peptide by chemical synthesis or by biotechnology using suitable protein expression vectors [54]. cDNA encoding morrisons have been identified in *M. sexta* [54] *Spodoptera litura* [55],*G. mallonella* [56], *H. armigera* [57], *S. exigua*, *virescens* and *Hyblaea puera* and *Bombyx mori*. From the results of molecular cloning, cDNA encoding moricin differ in the coding and non-coding regions which results to one amino acid substitution in the putative signal peptide efficient strategy in insects to protect themselves from bacterial invasion. Two dimensional 1H nuclear magnetic resonance (NMR) spectroscopy reveals the presence of a unique structure comprising of a α-helix containing eight turns along the entire length of the peptide except for four N-terminal residues and six C-terminal. Electrostatic surface map of amphiphatic N-terminal of the α-helix is important for

the increase in permeability of membrane to kill the bacteria [58].

4.4 Gloverins

G. Gloverin is a basic insect inducible antibacterial protein isolated from the pupae of giant silk moth *Hyalaphora cecropia*. It contains large number of glycine residues (18.5%) but no cysteine residues and has a distinct amino acid sequence that reveals no strong degree of identity with any known proteins [59]. Till now gloverin have been identified only in Lepidoptera including, *H. armigera* [60], *T. ni* [61], *G. mellonella* [62], *Antheraea mylitta* [63], *M. sexta* [64], *Diatraea acchariali*s [65], *S. exigua* [66], and *B. mori* [67]. Gloverins were found to be active against *E. coli*, mutant strains of (Df21f2, D21 and D22) containing Lipopolysaccharide (LPS). Gloverin from *T. ni* is active against virus and *S. exigua* is active against *Flavobacterium* sp., On the other hand it is inactive against *E. coli* strains with smooth LPS. Results of EST and whole genome shotgun analysis showed the presence of four gloverin like genes, BmGlv 1-4 homologous to *Hyalophora gloveri* gloverin. Northern Blot andRT-PCR analysis revealed that BmGlov genes have been induced in the fat body when challenged with *E. coli*, but the induction was less with yeast *Candida albicans.* In silico sequence analysis reveals that presence of a motif which is homologous to the Nuclear Factor κB (NF-kB) binding site in the upstream of each BmGlov gene. A recombinant form of BmGlov genes were also expressed in the Baculovirus virus system and found that all the BmGlov1-4 genes significantly inhibit the growth of *E. coli*. Bmgloverin 1 is considered as the ancestor among the four Bmgloverin genes, gloverin genes 2-4 are derived from the duplication. Knockdown expression studies of *B. mori* gloverin-2 performed using RNAi, showed reduction in the hatching rate of the embryos [68]. Bmgloverin 1 was found to be expressed only in larva but not in adult gonads; on the other hand gloverins 2-4 was expressed in adult but not in larval gonads [69].

4.5 Attacins

Attacins were first isolated from the hemolymph of bacteria immunized *H. cecropia* pupae having a molecular mass of 20-23 kDa. Attacins were divided into two groups basic (A-D) and acidic (E-F) having isoelectric points (pI 5.7 - 8.3). The different isoforms of attacin were generated by the post translational modifications of two parental pro-attacins sequences. It was reported that attacins have a very high content of aspartic acid, glycine, alanine and unusual levels of phenylalanine and threonine. Basic attacins reported to have a high content of threonine, glutamic acid, lysine and tryptophan whereas acidic attacins have more aspartic acid, isoleucine and arginine. Attacins were synthesized as pre-pro-proteins containing a signal peptide, a pro-peptide (P-domain), an N-terminal attacin domain, followed by two glycine-rich domains (G1 and G2 domains) [70]. A conserved motif of amino acids RXXR is present at the N-terminal pro-peptide of attacins [71] which is recognized by furin-like enzymes [72]. This finding indicates that mature attacins are produced by processing of pro-attacins by furin-like enzymes. Antibacterial assays of attacin It reveals that apart from *Escherichia coli Acinetobacter calcoacetius* and *Pseudomonas maltophilia* isolated from the gut of a Chineseoak silkworm were found to be sensitive to attacins. It was suggested that attacins act only on growing cells and cause chain formation, unlike cecropins which causes destruction of bacterial membranes. When cDNA of *B. mori* was hybridized with *Hyalophoa cecropia* attacin it was observed that cDNA consist of 846 nucleotides and it encodes attacin precursor protein. The mature peptide had 70.4, 68.3 and 18.8% indentity in aminoacid sequence with that of *Hyalaphora cecropia* acidic and basic attacins and *Sarcophoga peregrine* sarcotoxin IIA. The presence of two sub domains in the G domains in *B. mori, H. cecropia* attacins and *S. peregrine* sarcotoxin IIA, suggests that common amino acid residues in the subdomains are conserved during evolution and seems to play an important role in the activity of antibacterial proteins. The expression level of attacin quickly induced when injected with *Escherichia coli* cells into *B. mori* larvae and continued for 48 hours in the fat body and hemocytes [73]. The mechanism present in attacin which bring about the alteration in the structure and permeability of the outer membrane of *Escherichia coli* is related with specific inhibition of the synthesis of several outer membrane proteins including OmpC, OmpA, OmpF and LamB. The effect of inhibition is manifested by the reduction in the steady-state mRNA levels and as a minimum in part the result of block in transcription of the analogous genes. The transcription of the OmpC and OmpF genes is under the genetic control of the regulatory locus OmpB, composed by OmpR, encoding a cytoplasmic DNA-binding protein, and envZ, encoding a cytoplasmic membrane localized environmental sensor protein. In a mutant strain called HSK24, which have a deletion in ompR and envZ and has a mutated chromosomal OmpC on the other hand carries an intact ompC clone on the plasmid pHSK21 suggest that reduction in the amounts of OmpC and OmpA comparable to that was observed in wild-type strains after treatment with attacin. It was concluded that attacin effect on Omp genes requires neither OmpR nor envZ. These data suggest the presence of an unknown system in *E. coli* for the transcriptional regulation of a largeset of outer membrane proteins not known to be co-ordinately regulated instead attacin helps in the regulation of the system [74].

4.6 Lebocin

It is an antibacterial peptide rich in proline and O-glycosylated consisting of 32 amino acids isolated from by the haemolymph of silkworm, *B. mori* immunized with *Escherichia coli*. The glycosylation in the amino acid residue (15-Thr) is an essential feature for antibacterial activity. The primary structure and antibacterial activity of this peptide mainly coincides with abaecin [75]. Two different analogues of lebocin, lebocin 1 and lebocin 2 have indentical amino acid residues but they differ on length of their sugar chains on the threonine residues. It was reported that glycosylated threonines of these antibacterial proteins contain different sugars that is *N-acetylgalactosamine* and galactose in Lebocin1 and *N-acetylgalactosamine* in Lebocin 2. Amino acid sequence of lebocin3 showed one amino acid replacememt at 16 Leu and 15 Thr. Lebocin 4 amino acid sequence shows similarity with other members of lebocin. When lebocin was incubated with liposome preparation, it causes the leakage of entrapped glucose under low ionic conditions, which suggests that bacterial membrane is a target for lebocin. Biological significance of lebocin is still remains doubtful because it shows very weak antibacterial activity under physiological conditions and requires low ionic strength for full expression. Further, Lebocin 3 has combineeffect with cecropin D which suggest that antimicrobial protein works co-operatively in the immunity of *B. mori* [76].cDNA encoding

lebocin precursors have been identified in Lepidopteran species like *M. sexta* [77], *Trichoplusiani* [78], *Pseudoplusia includes* [79], *Pieris rapea* (Genbank accession number: JN587806), *H. viresecns* (genbank accession number: FJ546346), and *Antheraea peryni* (Genbank accession number: EU557311, EU57312 and DQ666499). All these lebocin precursors along with *Bombyx mori* precursors are proline rich peptides having 4 to 6 prolines residues present in N-termini of mature precursor protein. Precursors of *Bombyx mori* consists of extra 32-residue peptides with 7 prolines which are closed to the C-termini [80]. From the cotransfection experiment with the silkworm cell line, the overexpression of an erythroblast transformation-specific (Ets) family of protein called BmEts is essential for the elevation of activity of lebocin promoters. However, BmEts has no effect on cecropins 1, cecropins D, attacin and moricin promoters. BmEts activity towards lebocin promoter was found to be depends on at least two kB elements and the proximal GGAA/T motif located in the 5-upstream. In addition, it also collectively enhances the *E. coli* or BmRelish1-d2 stimulated lebocin promoter activation [81].

5. Conclusion

The severe problems related with multi drug resistant microorganisms have created crucial
demand for the development of alternative therapeutics. Concurrently with the increase in resistance to commercially available antibiotics, there is a serious need for novel, effective therapeutics with lesser or no side effects. However, AMPs are the promising candidate for the production of new generation antibiotics. At the industrial levels, many companies are concentrating on the development of AMPs atboth preclinical and clinical stages. These AMPs are produced by virtually all species as a part oftheir immediate non-specific defence. The worth of these peptides in clinical sectors consists oftheir broad spectrum activity; low propensity forresistance development, ease of synthesis butthe questions arises with their high cost, limitedstability. Several procedures are have been implemented to enhance AMPs with favourableactivity, ranging from the addition of non naturalamino acids and high-throughput screening formultimerization of linear sequences. Even though it is a known fact that resistance may evolve at any time bacterial populations are regularly exposed to elevated levels of AMPs, this concern should not discourage their development in near future.

6. References

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