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REVIEW

Antimicrobial peptides from freshwater invertebrate species: potential for future applications

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Abstract

Invertebrates are a significant source of antimicrobial peptides because they lack an adaptive immune system and must rely on their innate immunity to survive in a pathogen-infested environment. Various antimicrobial peptides that represent major components of invertebrate innate immunity have been described in a number of investigations over the last few decades. In freshwater invertebrates, antimicrobial peptides have been identified in arthropods, annelids, molluscs, crustaceans, and cnidarians. Freshwater invertebrate species contain antimicrobial peptides from the families astacidin, macin, defensin, and crustin, as well as other antimicrobial peptides that do not belong to these families. They show broad spectrum activities greatly directed against bacteria and to a less extent against fungi and viruses. This review focuses on antimicrobial peptides found in freshwater invertebrates, highlighting their features, structure-activity connections, antimicrobial processes, and possible applications in the food industry, animal husbandry, aquaculture, and medicine. The methods for their synthesis, purification, and characterization, as well as the obstacles and strategies for their development and application, are also discussed.

Keywords Antimicrobial peptide · Invertebrate · Freshwater · Immunity · Antibiotic · Aquatic environment

Introduction

Antibiotic resistant microorganisms have resulted through the overuse of antibiotic medications and chemical food preservatives, rendering previously effective antibiotics ineffective. According to available global estimates, drug-resistant pathogenic microorganisms caused 1.27 million human deaths in 2019 [1]. Antibiotic-resistant bacteria have necessitated the development of novel and effective natural antimicrobial agents that may be employed safely and effectively to attack pathogenic bacteria without causing antimicrobial resistance [2]. In the natural world, organisms coexist alongside infectious pathogens such as viruses, bacteria, fungi, and other parasites in their natural environments. Their evolutionary success in the diverse environments confirmed the occurrence of diverse, effective and broad-spectrum immune system [3, 4]. Various types of antibacterial compounds have been identified from prokaryotes and eukaryotes over the years. Antimicrobial peptides (AMPs), a broad set of natural proteins found in prokaryotes and eukaryotes that protect host organisms from invading pathogens [5, 6], are one of the promising natural antibiotics. The AMPs are endogenous peptide antibiotics with a mature peptide length of less than 100 amino acids, hydrophobic and cationic/basic characteristics, and sequence diversity. They adopt amphipathic structures such as α-helix, β-hairpin-like β-sheet, β-sheet, or α-helix/β-sheet mixed structures which are essential for their antimicrobial action. Such properties of AMPs are an adaptation that allows host organisms to live in a variety of settings that contain harmful microorganisms [7–10]. Antimicrobial peptides function by interacting with and permeating microbial membranes, making it more difficult for bacteria to build resistance to them than to traditional antibiotics [11]. Furthermore, most AMPs show bacterial cell selectivity over eukaryotic cells, making them effective against non-eukaryotic intruders [12, 13]. Some AMPs are known for their immuno-modulatory effects in addition to their antibacterial action [14, 15]. The invertebrate category, which is widely distributed, comprise

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over 80% of all known living animal species [16]. Invertebrates do not have an adaptive immune system, but they do have an effective innate immune system that protects them from harmful microbial invaders [15]. The AMPs have been found in a variety of invertebrate taxa, including terrestrial, marine, and freshwater invertebrates, and demonstrate a wide range of structure and genetic content (17–20). Although some have been found to originate as the processed form of other larger proteins, such as astacidin 1 from hemocyanin, they are synthesized through ribosomal translation of mRNA, as seen in all forms of life [8, 21, 22]. Antimicrobial peptides in marine invertebrates have been extensively examined [18, 23, 24], hence this review will not cover them. The focus of this review is on antimicrobial characteristics of AMPs isolated from freshwater invertebrate species, as well as structure-activity connections, antimicrobial mechanisms involved, and the potential for future applications in the food industry, agriculture, and medicine.

Table 1 Antimicrobial peptides characterised in freshwater invertebrates

Phylum/species	Antimicrobial Peptide	Tissue/Expression site	Antimicrobial spectrum	Reference
Annelida				
T. tessulatum	Theromacin	Large fat cells	G+	[33]
	Theromyzin	Large fat cells	G+	[33]
H. medicinalis	Neuromacin	Neurons and microglia	G+	[34]
	Lumbricin	Neurons and microglia	G+	[34]
	Theromacin	Large fat cells	ND	[35]
Crustacea				
P. leniusculus	Astacidin	Hemolymph	G+, G-, F	[21, 27]
	Crustin	Hemolymph	G+	[27, 44]
P. clarkii	Astacidin	Hemocyte and gills	G+, G-	[25, 26]
	Crustin	Hemocytes, intestine, heart, gills, stomach, hepatopancreas	G+, G-	[45]
C. quadricarinatus	Crustin	Hemocytes, gills, intestine, stomach, hepatopancreas	G+, G-, F	[50]
M. rosenbergii	Crustin	Muscle, gills and hepatopancreas	G+, G-	[46]
M. nipponense	Crustin	Hemocytes, heart, intestine, hepatopancreas, gills, stomach	V, B	[47]
E. sinensis	Crustin	Haemocytes, heart, gill, muscle, gonad, hepatopancreas	G+, G-, F	[48, 49]
Mollusca				
P. poeyana	Pom	Foot and visceral mass	G+, G-, F, V	[67, 68]
B. glabrata	Theromacin	Whole juvenile snails	ND	[36]
S. woodiana	Theromacin	Mantle, blood, gill, foot, liver, intestine	B	[37]
H. cumingii	Theromacin	Hemocytes, liver, foot, gill, adductor muscle, heart, mantle, intestine	G+, G-	[38]
	Defensin	Hepatopancreas, gills	ND	[57]
	Big Defensin	Mantle, blood, liver, kidney, stomach, intestine	ND	[63]
Arthropoda				
Aeschna cyanea	Defensin	Hemolymph	G+, G-	[55]
C. plumosus	Defensin A, B	Hemolymph	G+	[56]
Cnidaria				
H. vulgaris/ magnipapillata	Hydramacin	Endodermal epithelium	G+, G-	[39]
	Periculin	Endodermal epithelium, interstitial cells in ectoderm	G+, G-	[39]
	Arminin	Endodermal epithelium	G+, G-	[70]

G+, Gram-positive; G-, Gram-negative; F, Fungi; B, Bacteria; V, Virus; ND, Not determined

Antimicrobial peptides in freshwater invertebrates

Antimicrobial peptides are produced by a variety of freshwater invertebrate species. Some of the characterized antimicrobial peptide families such as astacidin, macin, crustin, and defensin, as well as the peptides theromyzin, lumbricin, arminin, periculin, Pom-1 and Pom-2 which do not belong to these families are reviewed.

Astacidin

Astacidins are cationic short peptides with less than 50 amino acid residues in their mature form. They are synthesized from precursors that have a highly conserved 22–23 amino acid signal peptide and a very varied C-terminal region. Some astacidins are derived from other larger proteins [21, 25, 26]. Astacidins have been isolated from crayfish species such as *Procambarus clarkii* and *Pacifastacus leniusculus* (Table 1). Mature astacidins from the two species have molecular weights of 1.8–3.9 kDa, with a theoretical isoelectric point of 9.4–11.8 kDa (Table 2). Two astacidins named astacidin 1 and astacidin 2 were isolated from *P. leniusculus* [21, 27]. Astacidin 1 was derived from the carboxyl-terminus of hemocyanin and forms β-structure (Fig. 1 A) in citric acid buffer at pH 4, 6, and 8 [21]. In contrast, the mature astacidin 2 from the same species, *P. leniusculus*, is a gene encoded linear peptide rich in proline/arginine [27]. Three isoforms of astacidin 1 (named *Pc*Ast1-a, -b, -c) from *P. clarkii* share high identity with astacidin 2 from *P. leniusculus* [25, 26]. Apart from astacidin 2 from *P. clarkii*, which lacks the GK amidation signal at the C-terminus, astacidins 1 and 3 have both the GK amidation signal and the signal peptide. In addition, transcriptomic screening indicated occurrence of astacidin-1, 2 and 3 in several species of Cambaridae, Astacidae and Parastacidae families. Astacidins-1,2 and 3 are encoded by a multi-genic astacidin gene family present in Astacoidea and Parastacoidea superfamilies [26]. The *P. clarkii* astacidins are also rich in proline/arginine residues and form simple linear structures as predicted from circular dichroism studies [26].

Antimicrobial susceptibility assays demonstrate that astacidins are effective against Gram-negative bacteria such as *Escherichia coli, Shigella flexneri, Acinetobacter baumannii, Vibrio anguillarum, Proteus vulgaris, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, as well as Gram-positive bacteria such as *Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus*, and *Micrococcus luteus* [21, 25–27]. In addition, the hemocyanin-derived astacidin 1 exhibited antifungal activity against *Candida albicans*, *Trichosporon beigelii*, *Malassezia furfur*, and *Trichophyton rubrum* [28]. Cationic antimicrobial peptides

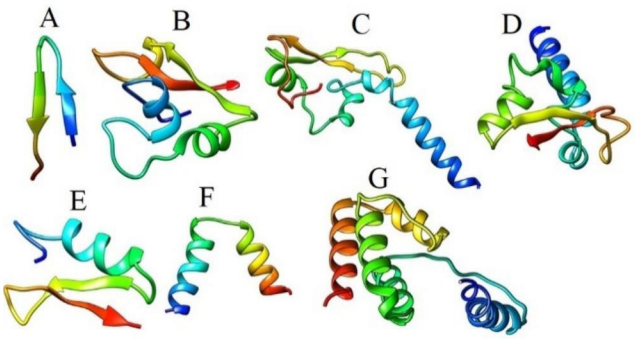

Fig. 1 The 3D-structures of selected antimicrobial peptides: **A**, *Pacifastacus leniusculus* astacidin 1; **B**, hydramacin-1 (PDB code 2k35); **C**, Theromacin (AF-Q6T6C2); **D**, neuromacin (AF-A8V0B3); **E**, *Aeschna cyanea* defensin; **F**, POM-2; **G**, Theromyzin (AF-Q6T6C1). Structure **A** was predicted using ITASSER and the corresponding sequence [21]. Structures **E** and **F** were predicted using Swiss-Model server and the corresponding sequences [55, 67]

interact with microbial cytoplasmic membranes via electrostatic forces between the positively charged peptide and the negatively charged phospholipids of the membrane bilayer (28,29). The interaction of a synthetic astacidin 1 from *P. leniusculus* with membranes of *C. albicans* and *T. beigelii* resulted into membrane damage via pore formation [28]. Formation of pores in cytoplasmic membranes permeabilizes and destroys membrane integrity (Fig. 2). Astacidin 1 was reported to cause membrane depolarization caused by an imbalance of K^+ movement across the cytoplasmic membranes of *C. albicans* and *T. beigelii*, consequently affecting K^+-dependent enzymes and/or pathways [28]. Similarly, the histidine-rich antimicrobial peptide PvHCt derived from hemocyanin of the marine shrimp *Litopenaeus vannamei*, exhibits antifungal properties by selectively binding and permeabilizing fungal cells [30]. Unlike astacidin 1 which forms β-structure, PvHCt adopts an amphipathic α-helical structure (21,28,30). Astacidin 2 and 3 possess a PRP motif similar to that found in human and insect proline-rich AMPs. Furthermore, *P. leniusculus* astacidin 2 (SLGYR**PRP**NYR**PRPIY**RPGK) has a PRPIY motif implicated in the reverse binding mode of pyrrhocoricin to DnaK, implying a relationship to DnaK binding [27, 31]. The PRPIY motif is absent from *P. clarkii* astacidin 2 (FY**PRP**YRPPYLPD**PRP**F**PRP**LPAFGHEFRRH), whereas the three *P. clarkii* astacidin 1 isoforms (*Pc*Ast1-a, b/c) have simple **RPxx** repetitions, such as **RP**AY**RP**AY**RP**SY**RP**GK in *Pc*Ast-1a. Proline-rich AMPs from mammals and insects have been found to enter Gram-negative bacteria like *E. coli* and *A. baumannii* via a specialized transporter called the SbmA membrane protein and bind to the bacterial chaperone DnaK, influencing the ATPase activity and/

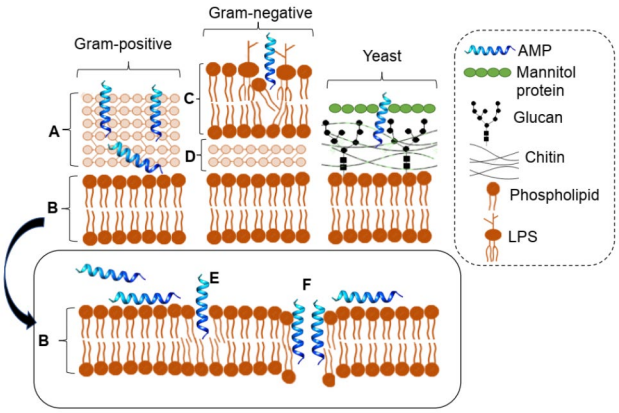

Fig. 2 Schematic illustration of the interaction of antimicrobial peptides with membranes of Gram-positive, Gram-negative, and fungi. In Gram-positive bacteria, AMPs have to cross the thick peptidoglycan layer (A) in order to reach the cytoplasmic membrane (B). In Gram-negative bacteria, AMPs have to penetrate the lipopolysaccharide (LPS) and phospholipids of the outer membrane (C), followed by crossing the thin-walled peptidoglycan layer (D). In fungi, AMPs must pass through mannitol proteins, glucans and chitin before reaching the cytoplasmic membrane. Electrostatic interactions between the cationic peptide and the negatively charged surface components such as LPS in Gram-negative, and teichoic acid (TA) in Gram-positive bacteria, are the first steps. Interestingly, LPS and TA are absent in membranes of multicellular animals. Moreover, the outer lipid monolayer of bacterial or fungal membranes consists of phospholipids such as phosphatidylserine and phosphatidylglycerol which are negatively charged, whereas animal membranes are made up of phosphatidylcholine, phosphatidyl ethanolamine, sphingomyelin and cholesterol which are neutral [73]. Such differences between the membranes of microbes and multicellular animals underpin AMP specificity for microbial membranes. Interaction with cytoplasmic membrane results in membrane permeabilization without pore formation (E) as occurs with hydramacin-1 or with pore formation (F) as occurs with most AMPs, killing the microorganism

or the peptide binding domain. As a result, protein folding activities are inhibited, and the bacterial cell is killed [31]. *S. aureus* has a DnaK homologue that does not bind pyrrhocoricin, which, combined with the observation that astacidins are active against both Gram-negative and Gram-positive bacteria lacking the SbmA membrane transporter, suggests the presence of alternative molecules or mechanisms that have yet to be discovered [32].

Macin

This category of antimicrobial peptides has been identified in leeches, bivalves, gastropods, and hydrozoans (33–39). It includes theromacin, neuromacin, and hydramacin (Table 1). Theromacins were identified in the species *Theromyzon tessulatum*, *Hirudo medicinalis, Sinanodonta woodiana*, *Hyriopsis cumingii*, *Biomphalaria glabrata, Hydra magnipapillata*, and *Hydra vulgaris* (Table 1). The macin peptides are synthesized as precursor molecules with a signal sequence (approximately 22–27 amino acid residues), signaling that they are secreted. In the species *H. cumingii*, theromacin transcripts were found to be constitutively expressed in a number of sites, with the highest level in hemocytes [38]. The putative mature theromacins contain 10 cysteine residues and show high similarity. In contrast, mature neuromacin and hydramacin contain eight cysteine residues, which are conserved across the macin family. Structural predictions indicate that hydramacin comprises of two α-helices at the N terminus separated by a long flexible loop, and two antiparallel β- strands similarly separated by a long flexible loop at the C-terminus [29]. These features are also reflected in the structures of theromacin and neuromacin (Fig. 1). Macins are more active against Gram-positive than against Gram-negative bacteria. For instance, purified theromacin from *T. tessulatum* was active against the Gram-positive bacteria *M. luteus*, with no activity against the Gram-negative bacteria *E. coli* or the fungus *Fusarium oxysporum* at the same concentration [33]. Moreover, purified neuromacin was also active against the Gram-positive bacteria *Micrococcus nishinomiyaensis* with no activity observed toward the Gram-negative bacteria *Aeromonas hydrophila* at the same concentration [34]. Neuromacin and theromacin are also reported to enhance repair of leech nerves [40]. However, hydramacin has been shown to be highly active against the Gram-positive bacteria *B. megaterium* and *Staphylococcus hemolyticus*, as well as the Gram-negative bacteria *Citrobacter freundii*, *Enterobacter cloacae*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Salmonella typhimurium*, and *Yersinia enterocolitica* [29, 39].

The bactericidal processes of the macin antimicrobial peptides can be thought of as starting with bacterial aggregation. Antimicrobial membrane binding investigations with hydramacin-1 and neuromacin, for example, revealed that bacterial aggregation is a first step in these peptides' killing mechanism [29, 40]. In hydramacin-1, the side chains of the arginine and lysine residues formed a positively charged belt that split the peptide's molecular surface into two hydrophobic hemispheres [29]. Michalek et al. showed that both the electrostatic and hydrophobic forces initiated the interaction between hydromacin-1 and bacterial membrane lipids [41]. The two hydrophobic patches of the peptide are introduced into the outer leaflets of two individual bacteria's membranes, which could explain the observed bacterial aggregation. The hydrophobic and electrostatic forces stabilize the peptide-lipid complex, promoting bacterial cytoplasmic membrane permeabilization and secondary intracellular events that lead to changes in cellular morphology without membrane pore formation [29, 41]. The ability of macins to permeabilize the bacterial membrane differs significantly. Neuromacin, for example, has a much greater ability to permeabilize the cytoplasmic membrane of *B.*

Table 2 Physicochemical properties of antimicrobial peptide precursors and mature peptides from freshwater invertebrates, predicted using ExPASy protparam tool [42]

AMP	Mw^p (kDa)	Th-pI	Charge	Mw^m (kDa)	Th-pI	Charge	Accession no. /reference
Macins							
*Hm*Neuromacin	9.3	8.4	+3	6.7	8.5	+3	ABW97519.1
*Tt*Theromacin	10.8	8.5	+4	8.5	8.6	+4	AAR12065.1
*Hc*Theromacin	10.4–10.8	6.2–7.5	-1 to +1	7.7–7.9	6.3–7.7	-1 to +1	ADK94899, AEC50045 ACQ90304
*Sw*Theromacin	11.2	8.8	+5	8.3	8.8	+5	AIA62156.1
*Hv*Hydramacin	9.7	9.3	+8	7.0	9.1	+6	ABE26989.1
Crustins							
*Pl*Crustin	12.3–16.5	7.4–8.5	+1 to +3	10.6–14.5	6.9–8.3	0 to +3	ABP88042-44
*Pc*Crustin	12.2–12.3	6.3–8.6	-1 to +4	10.4–10.6	5.9–8.5	-2 to +4	ACY64751-52
*Es*Crustin	11.6–12.4	6.8–8.4	0 to +2	9.3–10.5	6.2–8.3	-1 to +3	ACF25907-8
*Mr*Crustin	10.3–15.2	5.9–8.8	-2 to +6	8.2–12.8	4.9–8.7	-2 to +5	AFO68120, ANH22230-34, ABQ41250, AOF80301-02
*Mn*Crustin	12.5–25.0	4.5–8.4	+3 to +18	10.8–23.2	4.5–8.3	+2 to +3	QHG61850-51, QEQ76263, QIV66989, QNF22597-98
Astacidins							
*Pl*Astacidin	4.8	11.4	+7	1.8–1.9	10.0-11.8	+2 to +5	ABH05920, [21]
*Pc*Astacidin	4.8–6.3	9.8–11.6	+3 to +8	2.4–3.9	9.4–11.4	+2 to +7	QID92123-27
Defensins							
*Hc*Defensins	6.7-8.0	7.6–8.5	+1 to +3	4.3–7.1	8.3–8.7	+2 to +3	[57]
*Hc*Big defensin	12.6	8.5	+3	10.0	8.3	+2	AEP26934
Lumbricin							
*Hm*Lumbricin	6.6	9.2	+2	6.6	9.2	+2	ABW97520
Theromyzin							
Tt-Theromyzin	12.2	6.2	-4	10.0	6.0	-5	AAR12066

Hm, *Hirudo medicinalis*; *Tt*, *Theromyzon tessulatum*; *Hc*, *Hyriopsis cumingii*; *Sw*, *Sinanodonta woodiana*; *Hv*, *Hydra vulgaris*; *Pl*, *Pacifastacus leniusculus*; *Es*, *Eriocheir sinensis*; *Mr*, *Macrobrachium rosenbergii*; *Mn*, *Macrobrachium nipponense*; *Pc*, *Procambarus clarkii*; Mw^p, molecular weight of precursor peptide; Mw^m, molecular weight of mature peptide; Th-pI, Theoretical isoelectric point

megaterium than theromacin and hydramacin-1. Furthermore, only neuromacin showed pore-forming activity, and only in acidic conditions [29].

Crustin

Crustins are important antimicrobial peptides found in crustacean blood plasma and hemocytes, participating in the first line of the host immune system [27, 43]. Crustins have been identified in a variety of freshwater crustacean species, including *Macrobrachium rosenbergii*, *Macrobrachium nipponense*, *Procambarus clarkii*, *Pacifastacus leniusculus*, *Cherax quadricarinatus*, and *Eriocheir sinensis* (Table 1). Mature crustins in these species consist of more than 80 amino acid residues [27, 44–50] with calculated molecular masses of 8.2–23.2 kDa and isoelectric points of 4.5–8.7 (Table 2). The presence of eight conserved cysteine residues responsible for the formation of the four-disulfide core structure within the Whey Acidic Protein (WAP) domain at the C-terminal region is a feature of crustin AMPs [43]. The four-disulfide core structure provides the antimicrobial function as well as aid the peptides function as protease inhibitors [51–53]. Within the WAP region, there is a specific motif with the consensus sequence CXXDXX-CXXXXKCC typical of crustins suggesting a conserved function for this motif [27]. On the basis of differences in the amino acid sequences between the signal peptides and the WAP domains, crustins were divided into types I, II, and III [43]. In all the types, there is a signal peptide at the N-terminal, indicating they are secreted molecules. According to this classification scheme, type I crustin contains a cysteine-rich region between the signal peptide region and the WAP domain. In type II, there is a glycine rich profile followed by a cysteine rich profile between the signal peptide and the WAP domain region, while type III only contains a proline and/or arginine rich region between the signal peptide and the WAP domain. The majority of crustin peptides identified in freshwater crustaceans belong to one of the three crustin types [27, 44–48, 50]. The Double WAP Domain-Containing Protein, *Es*-DWD1 from *Eriocheir sinensis*, on the other

hand, does not belong to any of the three types, but to the DWD proteins and was assigned to the type IV subfamily containing dual WAP domain proteins [49]. This classification approach was expanded to type-V to include one of the novel ants crustins, resulting in five different types of crustins [19]. Type I and II crustins have not been reported to exhibit proteinase inhibitory activity. The type I recombinant *Pl*Crustin-1 and 2 tested against trypsin, chymotrypsin, elastase, and subtilisin A indicated no inhibitory activity against these enzymes [44]. In contrast, mature recombinant *Es*-DWD protein exhibits both antimicrobial and proteinase activities [49]. The antimicrobial activities of crustins indicate they are effective against bacteria, viruses, and fungi (Table 1). They have been shown to have antimicrobial activity against Gram-positive bacteria such as *M. luteus*, *M. tetragenus*, *B. subtilis*, *B. thuringiensis*, *B. megaterium*, and *S. aureus* [44–47], as well as Gram-negative bacteria such as *E. coli*, *P. aeruginosa, V. anguillarum*, *V. parahaemolyticus, V. alginolyticus*, and *A. hydrophila* [45, 47, 50] and the fungus *P. pastoris* [49, 50]. The antimicrobial mechanisms of these crustins have not been investigated. However, a type 1 recombinant crustin (rCrus1) from the marine shrimp *Rimicaris* sp., was found to bind to peptidoglycan and lipoteichoic acid, causing bacterial cytoplasmic membrane damage and membrane depolarization [54], highlighting the mechanism used by crustins to kill microorganisms.

Defensin and big defensin

Defensins have been identified in freshwater invertebrates such as the insects *Chironomus plumosus* (Order Diptera) and *Aeschna cyanea* (Order Odonata), as well as the triangle-shell pearl mussel *Hyriopsis cumingii* (Table 1). Mature peptides have a molecular mass of 3.8–7.1 kDa, are cationic, and comprise 36–60 amino acid residues [55–57]. Insect defensins have a lower molecular mass (3.8–4.1 kDa) than defensins found in the mussel *H. cumingii* (Table 2). Apart from *H. cumingii* defensin 2 (*Hc*Def2) with four conserved cysteine residues and the defensins *Hc*Def-1, 5 and 6 with eight cysteine residues, the rest of the defensins characterized in these species consist of six conserved cysteine residues [55–57]. In typical defensins, a central amphipathic α-helix is linked to an antiparallel double-stranded β-sheet by disulfide bridges, making the cysteine-stabilized α-helix/β-sheet motif (CSαβ) [58, 59]. Based on the disulfide bridges formed between the cysteine residues, defensins were categorised as α-(Cysl-Cys6, Cys2-Cys4, Cys3-Cys5), β- (Cysl-Cys5, Cys2-Cys4, Cys3-Cys6), θ-(Cysl-Cys6, Cys2-Cys5, Cys3-Cys4) and insect defensins (Cysl-Cys4, Cys2-Cys5, Cys3-Cys6) [60–62]. *Chironomus plumosus* and *A. cyanea* defensins have a three-dimensional structure that is typical of insect defensins [55, 56]. Only *Hc*Def3 and *Hc*Def4 in *H. cumingii* conform to the three-disulfide bonding array found in insect defensins, whereas *Hc*Def-1, 5, and 6 form four disulfide bonds due to the presence of an extra pair of cysteine residues [57]. The 8-cysteine pattern in *Hc*Def-1, 5 and 6 is similar to the type-8-cysteine defensins from the marine mussel *Mytilus galloprovincialis* and the nematode species *Ascaris suum* and *Caenorhabditis elegans* [19]. The CSαβ structural motif is comparable in insect defensins (type-6-cysteine) and mussel or nematode defensins (type-8-cysteine). They also have similar antibacterial action, demonstrating that three disulfide links are involved in biological activity, with the fourth disulfide bond in the matching defensins providing added stability [19]. In addition to the aforementioned defensins, a 113 amino acid precursor big defensin was isolated in *H. cumingii* (Table 1). The precursor and mature forms of the big defensin peptide are cationic with predicted molecular masses of 12.6 kDa and 10 kDa, respectively (Table 2), and contain a trans-membrane domain, a hydrophobic region, and alpha helices [63]. The *H. cumingii* big defensin was shown to have six conserved cysteine residues, producing a consensus pattern of C-X6-C-X3-C-X13(14)-C-X4-C-C and a disulfide array of Cys1-Cys5, Cys2-Cys4, Cys3-Cys6 typical of beta defensins. This disulfide bonding array is not seen in the defensins of *C. plumosus*, *A. cyanea*, or *H. cumingii*, but it is found in other big defensins [63].

The antimicrobial profiles indicate that invertebrate defensins are more active against Gram-positive bacteria than Gram-negative bacteria, a typical characteristic of CSαβ-containing defensins [62]. For instance, *A. cyanea* defensin is stronger against Gram-positive than against Gram-negative bacteria [55]. Similarly, the two defensins from *C. plumosus (Cp*Def-A and B*)* were active against Gram-positive bacteria [56]. Insect defensins are known to form channels in bacterial lipid membranes, resulting into membrane permeability, potassium ion leakage and the induction of membrane depolarization [64]. Unlike classical defensins which permeabilize both the outer and inner membranes of Gram-negative bacteria, most insect defensins may not be capable to permeabilize both the outer and inner membranes of Gram-negative bacteria [64]. This may account for the selective activity of *A. cyanea* and *C. plumusus* defensins against Gram-positive bacteria. This high antimicrobial activity of defensins directed against a wide range of Gram-positive bacteria and a few Gram-negative bacteria has also been observed in mollusc and nematode defensins [19, 65]. Although this may suggest related bacterial killing mechanisms, the recombinant oyster defensins named *Cg*-Defh1, *Cg*-Defh2, and *Cg*Defm were reported to bind lipid II, a peptidoglycan precursor, after it has been translocated to the outer leaflet of the cytoplasmic membrane, resulting in inhibition of cell wall biosynthesis without causing

cytoplasmic membrane damage [66]. Therefore, the differential access to lipid II, which is also easily accessible in Gram-positive bacteria but would require outer membrane damage to become accessible in Gram-negative bacteria, may account for the selective activity of oyster defensins against Gram-positive bacteria as well [66].

Other antimicrobial peptides

Peptides such as theromyzin from the leech *T. tessulatum* and lumbricin from *H. medicinalis* form α-helical linear structures. Precursor theromyzin contains a signal peptide, while lumbricin lacks the signal peptide, although both are reported to be secreted peptides [33, 34]. In addition, theromyzin is an anionic peptide, while lumbricin is cationic (Table 2). Due to the concentration of histidine residues at the N-terminal part of theromyzin, it has been suggested that this terminal part enriched with histidine and aspartate residues could be involved in antimicrobial activity, often requiring a cofactor. Theromyzin was reported to be active against the Gram-positive bacteria *M. luteus* [33]. In the freshwater snail *Pomacea poeyana*, antimicrobial peptides designated Pom-1 and Pom-2, each containing 34 amino acid residues, were identified [67]. Sequence comparison with known peptides indicates that Pom-1 is a fragment of Closticin-574 while Pom-2 is a fragment of cecropin D-like peptide first isolated from *Galleria mellonella* hemolymph [67]. Both Pom-1 and Pom-2 form structures with two α-helices, which in Pom-1 are connected by a six amino acid loop, while in Pom-2 the α-helices are connected by three amino acids [67, 68]. They show antibacterial, antifungal and antiviral activities with varying potency. For instance, high activity was observed with Pom-1 against *P. aeruginosa* and moderate activity against *K. pneumoniae* and *Listeria monocytogenes*. In addition, Pom-1 moderately inhibited Zika Virus infection but slightly enhanced HIV 1 infection invitro [67]. Both Pom-1 and Pom-2 are reported to moderately inhibit planktonic forms but highly inhibited biofilm formation in *C. albicans*, *C. parapsilosis* and *C. auris* [68]. The structures of both Pom-1 and Pom-2 are similar to those of cecropins, suggesting similarities in their microbial killing mechanisms, which may involve disruption of microbial membranes (Fig. 2) following a carpet model as reported for cecropins [67, 69].

Other novel antimicrobial peptides include periculin and arminin expressed in endodermal epithelium of hydra species [39, 70]. In both peptides, there is a negatively charged N-terminal region and a positively charged C-terminal region. Expressed sequence tags and genome-wide sequence analysis using *H. magnipapillata* resulted into three classes of arminin namely arminin class 1 (1-a, b and c), arminin class 2 (2-a, b and c) and arminin class 3 (3-a and b) [70]. The C-terminal part of arminin 1a (c-arminin 1a) contains 31-amino acids, adopts an α-helix structure, and exhibits potent and broad-spectrum antimicrobial activity [70, 71]. At concentrations of 0.1–1.6 µM, c-arminin 1a exhibited strong bactericidal activity against *B. megaterium* ATCC 14,581, *E. coli* DH5α, *S. aureus* ATCC 12600, methicillin-resistant *S. aureus* (MRSA), the vancomycin-resistant *Enterococcus faecalis* and *Enterococcus faecium*, and the ESBL-producing *K. pneumoniae* and *E. coli* strains. In addition, c-arminin 1a was observed to destroy and detach the peripheral cell wall of *S. aureus* ATCC 12600 in a similar manner to the effects of human beta defensin 3 (HBD3) on *S. aureus* cells, suggesting similarities in bacterial killing mechanisms involving cell wall biosynthesis machinery [70, 72]. C-arminin 1a has also been shown to destroy leukemia cells by forming membrane pores [71].

Purification, characterization and synthesis of AMPs

Extraction, purification, and characterization are typical procedures for the discovery of antimicrobial peptides. The AMPs are usually isolated from their natural sources, such as invertebrates, upon induction by pathogens, resulting in AMP production, which is then extracted, purified, and characterized. During extraction and purification processes, the bioactive fractions are usually identified by subjecting them to antimicrobial activity assays. In addition to natural peptide extraction, genetic and in silico techniques are employed in AMP discovery, offering simplicity and reducing laboratory costs associated with the natural peptide extraction approach. In the genetic approach, ESTs or cDNA sequences are analyzed for sequences showing similarity to known AMP sequences, which are then isolated and synthesized. In the in-silico method, ESTs or cDNA, and genome databases are searched for the presence of potential sequences based on known structural features of AMPs like charge and hydrophobicity using computational tools [24]. The AMPs can then be synthesized in the laboratory and subjected to bioactivity assays. Three methods employed in peptide synthesis include chemical synthesis, enzymatic synthesis, and biosynthesis (recombinant DNA technique) [74–76]. Chemical synthesis is the most common method employed in peptide synthesis which can occur via solid-phase or solution-phase peptide synthesis [74, 75]. A number of purification techniques such as dialysis, ultrafiltration, and chromatography are employed (Table 3). Chromatography techniques such as solid phase extraction on C18 cartridges, size exclusion/gel filtration chromatography, affinity chromatography, ion-exchange chromatography, and reversed-phase high-performance

Table 3 Methods used in discovery, purification and characterization of antimicrobial peptides from freshwater invertebrate species

Source organism	AMPs	Discovery method	Production method	Purification method(s)	Characterisation method(s)	Reference
Theromyzon tessulatum	Theromacin, Theromyzin	Bioassay-guided purification	Extraction from source organism	1. SPE 2. RP-HPLC	1. ESI- MS 2. MALDI TOF-MS 3. Edman degradation	[33]
Hirudo medicinalis	Neuromacin, Lumbricin	Bioassay-guided purification	Extraction from source organism	1. SPE 2. RP-HPLC	MALDI TOF-MS	[34]
Pacifastacus leniusculus	Astacidin 1	Bioassay-guided purification	Extraction from source organism	1. SPE 2. IEC (cat.) 3. RP-HPLC	1. Acid-urea PAGE 2. Edman degradation 3. MALDI TOF-MS 4. CD spectrum	[21]
	Astacidin 2	Bioassay-guided purification	Extraction from source organism	1. SPE 2. IEC (cat.) 3. RP-HPLC	1. Acid-urea PAGE 2. Edman degradation 3. MALDI-TOF-MS	[27]
	Crustin	Genetic	Expression in *E. coli*	1. AFC 2. Dialysis	SDS-PAGE	[44]
Procambarus clarkii	Astacidin (*Pc*Ast-1a)	Genetic	Chemical synthesis	NA	NA	[25]
	Astacidin (1b, 1c,2,3)	Genetic	Chemical synthesis	RP-HPLC	1. MS 2. CD	[26]
	Crustin	Genetic	Expression in *E. coli*	1.AFC 2.Dialysis	SDS-PAGE	[45]
Macrobrachium rosenbergii	Crustin	Genetic	Expression in *E. coli*	1. AFC 2. Dialysis	1. MS/MS 2. SDS–PAGE	[46]
Eriocheir sinensis	Crustin	Genetic	Expression in *E. coli*	AFC	1. SDS-PAGE 2. MS	[48, 49]
Pomacea poeyana	Pom-1 and 2	In silico	Solid phase synthesis	1. Ultrafiltration 2. RP-HPLC	1. UPLC-ESI-MS/MS 2. LC-MS 3. CD	[67]
Aeschna cyanea	Defensin	Bioassay-guided purification	Extraction from source organism	1. SPE 2. RP-HPLC 3. SEC 4. RP-HPLC	1. Edman degradation 2. ESI-MS	[55]
Chironomus plumosus	Defensin A, B	Bioassay-guided purification	Extraction from source organism	1. SPE 2. SEC 3. RP-HPLC	1. Edman degradation 2. MALDI-MS	[56]
Hydra vulgaris/ magnipapillata	Hydramacin	Bioassay-guided purification	Extraction from source organism	1. Dialysis 2. AFC 3. RP-HPLC 4. CE-HPLC 5. RP-HPLC	1. SDS–PAGE 2. ESI-MS 3. MS/MS	[39]

AFC, Affinity chromatography; IEC, Ion exchange chromatography; RP-HPLC, Reversed-phase high performance liquid chromatography; CE-HPLC, cation exchange HPLC; SEC, size-exclusion chromatography; SPE, Solid Phase Extraction; CD, circular dichroism; ESI-MS, Electrospray Ionization Mass Spectrometry; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization-Time-Of-Flight; NMR, Nuclear Magnetic Resonance; SDS-PAGE, Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis; NA, Not Available

liquid chromatography (RP-HPLC) are commonly used. Garcia et al. obtained the low-molecular-weight peptide fraction containing Pom-1 and 2 peptides from *Pomacea poeyana* homogenized samples using ultrafiltration with a cut-off of 10 kDa [67]. This ultrafiltration process can be used to concentrate peptides by removing superfluous proteins and peptides beyond the target peptide's molecular size range. Solid phase extraction is also commonly used to clean up and concentrate the analyte thereby simplifying the downstream procedures. Chromatography mainly on the reversed-phase column is the principal mode of antimicrobial peptide purification (Table 3). In the majority of investigations, RP-HPLC is the most widely used technique for the final purification of antimicrobial peptides (Table 3). Due to its high resolving power, RP-HPLC can be utilized to generate peptide fractions based on their hydrophobic characteristics and is appropriate for the purification of a wide range of antimicrobial peptides. In some purification schemes, both ion exchange chromatography and reversed-phase chromatography have been used (Table 3).

Characterization of purified peptides is mainly performed using techniques such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight approximation, sequencing by automated

Edman degradation, mass spectrometry analysis, and enzymatic cleavage [77]. Due to the high speed, sensitivity, and specificity of the technique, mass spectrometry is used in mass analysis and for confirming amino acid sequences of peptides [78]. Liquid chromatography can be combined with mass spectrometry or followed by tandem mass spectrometric detection (LC–MS/MS) to characterize peptides. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) have been applied to create ionized analytes for acceleration to the analyzer (Table 3). For instance, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), as well as MALDI-TOF/TOF MS, have been used for generating peptide profiles of protein hydrolysates (Table 3). Furthermore, ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis with high throughput and reduced analysis costs has been used for peptide purification and characterization [67]. The characterization of the 3D peptide structure can be examined in solution using circular dichroism (CD) and nuclear magnetic resonance (NMR) techniques. The structural integrity of chemically synthesized or recombinant proteins can be predicted or confirmed using the CD technique. For instance, Lee et al. used CD to predict the two-β-sheet secondary structure of astacidin 1 [21]. Circular dichroism can also be a necessary step before subjecting proteins to detailed structural determination by high-resolution techniques such as NMR Spectroscopy and X-Ray crystallography [79].

Potential for application of AMPs from freshwater invertebrate species

Freshwater invertebrate AMPs may have potential for applications in animal husbandry, aquaculture, food preservation, and medicine because they have been reported to be effective against bacteria, fungi and some viruses (Table 1). While some AMPs show broad spectrum activities, others are more effective against Gram-positive bacteria and vice versa. The AMPs can therefore be used either alone or in combination to combat pathogenic and foodborne microbes, and this will require that studies investigating their efficacy in animal husbandry, aquaculture, food preservation, and therapeutics be initiated in order to realise their potential.

Animal production

In poultry, pathogens including the Gram-negative avian pathogenic *E. coli*, *Salmonella pullorum, Salmonella gallinarum*, *Pasteurella multocida*, *Avibacterium paragallinarum*, *Gallibacterium antis*, *Ornitobacterium rhinotraceale*, *Bordetella avium*, *Riemerella anatipestifer*, and Gram-positive bacteria including *Chlostridium perfringens*, *Mycoplasma* spp., *and Erysipelopathiae*, affect production [80]. In ruminants, *Campylobacter jejuni* and *Campylobacter coli* cause intestinal campylobacteriosis, while *Pasteurella multocida* and *Mycoplasma mycoides* are responsible for hemorrhagic septicemia and bovine pleuropneumonia, respectively. In addition, *Mycoplasma pneumoniae* causes respiratory disease and arthritis in cattle, while bovine brucellosis caused by *bovine abortus* is a cause of abortion in cows [81]. Most of these pathogens are already showing resistance to available antibiotics used for treatment of these infections. For instance, avian pathogenic *E. coli*, *S. pullorum/gallinarum*, *M. gallisepticum*, and *G. anatis* are showing increasing resistance to ampicillin, amoxicillin, and tetracycline [80]. Antimicrobial peptides can therefore be seen as excellent alternatives to conventional antibiotics as growth promoters and preventive agents of infectious diseases in animal production [82]. The use of swine defensin and fly antimicrobial peptide as feed additives for young goats resulted in benefits such as an increase in body weight, average daily weight growth, and enzymatic activity, as well as a greater diversity of rumen microorganisms [83]. Antimicrobial peptides were found to be effective in promoting growth, preventing disease, and lowering death rates in broiler chickens [84], and had a similar effect to conventional antibiotics in improving growth performance, digestibility, small intestine morphology, and blood serum parameters [85].

Aquaculture

Gram-negative bacterial pathogens like *A. hydrophila*, *A. salmonicida*, *Vibrio* spp, *Edwardsiella ictaluri*, *E. tarda*, and Gram-positive bacteria like *Streptococcus* spp., as well as the water mould *Saprolegnia* spp., affect aquaculture species [86–88]. Antimicrobial peptide crustin from the crayfish *P. clarkii* protected crayfish from infection by the pathogenic bacteria *A. hydrophila* in vivo [46], demonstrating potential for application in aquaculture. Similarly, broad-spectrum antimicrobial peptides including astacidin, crustin, hydramacin, and pom-1 are candidates for application in aquaculture. Dietary supplementation of recombinant piscidin AMP led to the improvement of growth, oxidation resistance, immunity, increase in digestive enzyme activities and intestinal morphology of fish [89]. Production of recombinant AMPs from freshwater invertebrates and evaluation of their performance in promoting growth, preventing disease, and effects on intestinal microbiota will be important in highlighting their potential in aquaculture.

Food preservation

A number of foodborne pathogens, including *Salmonella* spp., *Listeria monocytogenes*, *S. aureus, Bacillus cereus, Clostridium botulinum, Clostridium perfringens, Shigella* spp., *Vibrio* spp., and *campylobacter* spp., widely occur and are associated with food contamination [90]. Other bacteria, including *A. salmonicida, B. thermosphacta*, *Pseudomonas fluorescens*, *P. fragi, Shewanella liquefaciens*, and *S. putrefaciens*, are responsible for food spoilage, resulting in large economic losses [91, 92]. *Brucella melitensis*, *Campylobacter* spp., *Listeria* spp., *Salmonella* spp., Shiga-toxin-producing *E. coli*, *S. aureus*, and *Toxoplasma gondii* have all been linked to dairy product contamination [93]. Their growth on food is frequently associated with corresponding changes in the color, odor, taste, or texture of products, resulting in waste and a negative impact on the industry. As a result, it is critical to consider microbial safety and spoilage prevention of food products, which will necessitate the use of novel and highly safe preservation methods involving the use of natural biocidal agents such as antimicrobial peptides that can function under a variety of food storage conditions [94]. Antimicrobial peptides make good candidates for application in the preservation of such products because a number of AMPs have been proven to inactivate these pathogenic bacteria. For instance, high activity of the antimicrobial peptide, Pom-1, against *P. aeruginosa* and moderate activity against *L. monocytogenes* was reported [67].

Human medicine

Many diseases affecting humans are caused by bacteria, fungi, and viruses, with some of the strains already resistant to available antibiotic drugs and claiming human lives [1]. In order to deal with the morbidity and mortality caused by fungal, bacterial, and viral diseases, the development of more effective antimicrobial agents is necessary. Antimicrobial peptides such as astacidins have been shown to be effective against fungi and bacteria [21, 25–28]. For instance, astacidin-1 exhibited antifungal activity against *C. albicans*, *T. beigelii*, *M. furfur*, and *T. rubrum*. Astacidin-1 also exhibited fungal cell selectivity in human erythrocytes without causing hemolysis [28]. Other astacidins, notably from *P. clarkii*, exhibited antimicrobial activity against the antibiotic-resistant clinical isolates of both *E. coli* and *A. baumannii* [26]. In addition, Pom-1 and Pom-2 inhibited both the planktonic and biofilm forms of *C. albicans*, *C. parapsilosis*, and *C. auris* [68]. Synthetic Pom-1 also showed high antimicrobial activity against *P. aeruginosa*, one of the leading causes of nosocomial infections and the cause of morbidity and mortality in cystic fibrosis patients [67]. Moreover, hydramacin-1 from *H. magnipapillata* exhibited antimicrobial activity against a broad spectrum of microbes of clinical importance. For instance, hydramacin-1 was active against *E. cloacae* and multi-resistant *K. oxytoca* without showing cytotoxic effects on human erythrocytes [29]. Cotton fibers coated with hydramacin-1 and lysozyme also inhibited the development and colonization of Gram-positive *B. subtilis* and Gram-negative *E. coli*, suggesting that it can be improved and used in medical cotton-based materials [95]. Moreover, c-arminin 1a, also isolated from hydra species, exhibited broad-spectrum antimicrobial activity against multi-resistant human pathogenic strains, such as the methicillin-resistant *S. aureus* strains [70].

Challenges and strategies for antimicrobial peptide development and application

Despite the observation that the majority of antimicrobial peptides studied have broad spectrum activities with no harmful effects on human cultured cells at effective doses [26, 28, 67, 70, 71], their stability and target selectivity are questionable under challenging physiological conditions. For instance, while c-arminin 1a was active in the presence of a wide range of salt concentrations (0–200 mM sodium chloride) [70], three synthetic astacidins (*Pc*Ast-1a, -1b/c, and-2) exhibited different antimicrobial activity and potency under different concentrations of the Mueller Hinton medium, indicating the influence of nutrient and salt concentrations in the medium [26]. In nature, apart from the amino acid glycine, which does not have a chiral center, the rest of the amino acids naturally exist in the L and D isomeric forms. As the L-amino acids are predominant in living organisms, the AMPs produced have a limited number of D-amino acids, making natural AMPs highly susceptible to protease degradation and rapid kidney clearance [96]. They also show high sensitivity to pH and temperature and can be toxic to the host cells. These shortfalls indicate that not all antimicrobial peptides are readily available for use, as they may require some form of modification in order to withstand the challenging conditions of the host and bacteria while preserving and/or improving antimicrobial efficacy.

In order to increase the stability, selectivity, and efficacy of AMPs, approaches such as chemical modifications of AMPs and the use of delivery carriers have been suggested. Chemical modification approaches, including isomerization of L-amino acids to the D-amino acids, addition of unnatural amino acids to the AMPs, peptide lipidation, multimerization, peptidomimetics, and cyclization of AMPs, have been applied to peptides from different sources [76, 96, 97]. Isomerization of amino acids from L-to-D enantiomers enhances the proteolytic stability of AMPs. For example,

after isomerization of the RR4 peptide, the D-enantiomer improved its antimicrobial activity against multidrug-resistant strains of *P. aeruginosa* and *A. baumannii* while retaining its high antibacterial and low hemolytic activities under challenging physiological conditions of high salts and acidic pH [97]. Chemically synthesized unnatural amino acids can also be incorporated into the peptide to achieve stability against proteases. The unnatural amino acids provide more net positive charge and bulky side chain groups to the peptide, enhancing peptide binding to microbial membranes and resistance against proteolytic degradation [98]. The AMPs can also be lipidated by attaching fatty acid chains to the amine groups of the N-terminus or the lysine residue of antimicrobial peptides. The improvement in antibacterial properties and selectivity depends on the length of the acyl chain, with acyl chain lengths of 8–12 carbon atoms reported to be more effective [96]. Linear antimicrobial peptides can also be dimerized and cyclized by joining their backbone N- and C-termini or by disulfide bridges to improve their stability and selective toxicity. The backbone cyclized KR-12 dimers with linkers of two to four amino acid residues showed improved antimicrobial activity and stability compared to the monomeric KR-12 form [99].

Other strategies involve the use of peptidomimetics and delivery systems. In peptidomimetics, the peptide backbone is modified while conserving the 2D and 3D spatial arrangement of the peptide side chains to maintain antimicrobial activity. This modification may involve bonding the side chain to the backbone nitrogen instead of the alpha carbon to make the peptide resistant to protease degradation as used to generate peptoids [73]. Delivery nanostructures such as mesoporous silica, titanium dioxide, metal nanoparticles (e.g., Au and Ag), graphene, quantum dots, carbon nanotubes, lipid-based nanostructures, polymer-based nanostructures, and tetrahedral framework nucleic acids are used in controlled drug delivery systems. They are also useful in the active packaging industry to preserve food. The use of tetrahedral framework nucleic acids has been reported to be suitable for AMPs with potent antimicrobial activity but cytotoxic to the host cells. The attachment of AMPs to the delivery systems can either be through covalent bonding or non-covalently by encapsulating them in the delivery systems. The use of nanostructure delivery systems improves the stability, target selectivity, half-life, bioavailability, and pharmacodynamics of AMPs by inhibiting renal clearance and enhancing retention and permeability of AMPs [73, 76, 100].

Conclusions

The excessive use of antibiotic drugs and the corresponding emergence of antibiotic-resistant microbial strains has led to the search for natural bioactive compounds such as antimicrobial peptides that can be used to overcome the burden of antibiotic resistance. In freshwater invertebrates, antimicrobial peptides have been identified in the phyla Arthropoda, Annelida, Cnidaria, Crustacea, and Mollusca. They form amphipathic structures ranging from linear to mixed α-helix/β-sheet structures, implying a variety of microbial pathogen-killing mechanisms. However, only a few of these antimicrobial peptides, notably some members of the macin and astacidin families, have been subjected to detailed studies of their microbial killing mechanisms. Some of the antimicrobial peptides are highly effective against multi-resistant bacterial strains pathogenic to humans and show no toxic or hemolytic effects on human cultured cells at effective concentrations. Such potent and broad-spectrum antimicrobial peptides offer promising templates for new classes of antibiotics. Approaches to antimicrobial peptide extraction, purification, characterization, and synthesis, including their modifications to overcome the shortfalls that would limit their application, are increasingly becoming well established. The evaluation of antimicrobial peptide performance in animal husbandry, aquaculture, food preservation, and medicine, as well as the production of their synthetic versions with enhanced stability, target selectivity, and efficacy, will be critical in highlighting their potential applications in such fields.

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