



# The potential of antimicrobial peptides isolated from freshwater crayfish species in new drug development: A review



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## ABSTRACT

The much-publicised increased resistance of pathogenic bacteria to conventional antibiotics has focused research effort on the characterization of new antimicrobial drugs. In this context, antimicrobial peptides (AMPs) extracted from animals are considered a promising alternative to conventional antibiotics. In recent years, freshwater crayfish species have emerged as an important source of bioactive compounds. In fact, these invertebrates rely on an innate immune system based on cellular responses and on the production of important effectors in the haemolymph, such as AMPs, which are produced and stored in granules in haemocytes and released after stimulation. These effectors are active against both Gram-positive and Gram-negative bacteria. In this review, we summarise the recent progress on AMPs isolated from the several species of freshwater crayfish and their prospects for future pharmaceutical applications to combat infectious agents.

## 1. Introduction

Innate immunity represents the first line of defence against invading pathogens and is conserved throughout the evolution of multicellular organisms (Akira et al., 2006). In crustaceans, innate immunity is based on the closely-related humoral and cellular immunity pathways (Hoffmann et al., 1999; Lin and Söderhäll, 2011; Fredrick and Ravichandran, 2012). Cellular immunity relies mainly on phagocytosis, encapsulation and haemocyte nodulation (Jiravanichpaisal et al., 2006; Vazquez et al., 2009) whereas, humoral immunity is linked to haemolymph agglutination, activation of the prophenoloxidase (proPO) system, the production of reactive intermediates of oxygen and nitrogen, and of lectins and of AMPs (Söderhäll and Cerenius, 1998; Wu et al., 2009, 2019; Wongpanya et al., 2017). Antimicrobial peptides (AMPs) are a group of molecules which are considered a major component of the innate immune defence system of all living organisms, ranging from bacteria to mammals, including fungi and plants. These bioactive molecules are primarily known as “natural antibiotics”, due to their rapid and efficient activity against a wide spectrum of microorganisms, ranging from Gram-positive and Gram-negative bacteria to viruses and parasites (Yount et al., 2006; Guani-Guerra et al., 2010), thus participating in the neutralization and elimination of foreign pathogens (Zasloff, 2002; Wu

et al., 2019). The AMPs recently extracted for the first time are cecropins, obtained in particular from the haemolymph of the giant silk moth, *Hyalophora cecropia* (Hultmark et al., 1980). This AMP family consists of both functional and pseudo-genes, being classified into five subtypes (cecropin A-E) that vary between species, with many of these having been characterized with in various insects (Peng et al., 2019). More than 3000 AMPs, both synthetically-produced and isolated directly from organisms, are included in specific databases such as the Antimicrobial Peptide Database (APD3, expanded version of the original APD) (Wang et al., 2015a), the Collection of Antimicrobial Peptides (CAMP3) (Waghu et al., 2015) and the Linking Antimicrobial Peptides (LAMP) database (Zhao et al., 2013). Over the last decades, scientific research has paid increasing attention to these natural antibiotics due to their promising role as therapeutic drugs (Giuliani et al., 2007; Hadley and Hancock, 2010; Hughes and Fenical, 2010). In fact, the rapid increase in drug-resistance observed in pathogens worldwide and the failure of most conventional antibiotics to counteract “superbugs” has focused attention on the urgent need to discover new alternative antimicrobials (Bahar and Ren, 2013). Compared to antibiotics, AMPs present a number of advantages: i) they are less stable in the environment and thus have less potential to bioaccumulate; ii) they demonstrate multiple mechanisms of action, disrupting bacterial cell membranes and

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inhibiting their metabolic pathways and iii) they do not increase bacterial mutation rates (Rodríguez-Rojas et al., 2014). Generally, AMPs are described as small, amphipathic, gene-encoded oligopeptides (<10 kDa, 15–100 amino acids) that show different amino acid compositions and structural conformations, which in turn determine their mechanism of action (Rosa and Barraco, 2010). AMPs are classified according to their secondary structure into  $\alpha$ -helical,  $\beta$ -sheet or peptides, with extended/random coil-structure (Takahashi et al., 2010; Nguyen et al., 2011; Pasupuleti et al., 2012). The major part of AMPs interacts with microorganisms by disrupting the membrane integrity through two different ways: i) the electrostatic forces between the cationic AMPs and the negatively-charged bacterial surface; ii) the formation of amphipathic structures in a hydrophobic environment that allows AMPs to penetrate into the bacterial phospholipid bilayer (Sperstad et al., 2011; Mahlapuu et al., 2016). The mechanism of action of AMPs is based on two different strategies: i) a membrane-disruptive strategy through which the peptides bind to bacterial membrane and kill rapidly the microorganism, creating irreversible pores or destabilizing the membrane through an efflux of cytoplasm; ii) a membrane non-disruptive strategy, through which some peptides inhibit bacterial growth by crossing the membrane and binding to intracellular components, as observed in several proline-arginine peptides such as pyrrhocoricin, bactenecin-7, apidaecin and drosocin. This strategy is considered more selective towards microorganisms, due to the interference of AMPs with stereospecific receptor/docking molecules, probably a component of the permease-type transporter system, followed by the translocation of the peptide inside the cell, where it interacts with DNA and protein synthesis, limiting the release of endotoxins (Nicolas, 2009; Sperstad et al., 2011). Beyond this direct interaction with microbial membranes, several studies reported that AMPs can interfere with intracellular targets and with protein, nucleic acid and cell wall synthesis, leading to bacterial cell death (Kamysz et al., 2003; Brogden, 2005; Yount et al., 2006; Hale and Hancock, 2007; Nicolas, 2009). In literature, there are various studies demonstrating not only how aquatic organisms play a very important role as bioindicators to evaluate the effects of different stressful conditions (Cordero et al., 2016; Celi et al., 2013, 2015; Nicosia et al., 2014; Parisi et al., 2017; Mauro et al., 2020a; Chiaramonte et al., 2020; Vazzana et al., 2020a, b), but also an even more important role as a source of bioactive molecules and for improving the understanding of human diseases (Lazzara et al., 2019; Inguglia et al., 2020; Mauro et al., 2020b, 2021; Luparello et al., 2020a, b). Among invertebrates, crustaceans in recent years have emerged as an important source of bioactive molecules with novel pharmaceutical properties. As in all invertebrates, even in crustaceans, the lack of a specific adaptative immune system, based on lymphocytes, has led to the evolution of effective molecules such as AMPs (Rosa and Barraco, 2010; Cerenius and Söderhäll, 2018). A major part of these molecules is produced after an immune stimulation and these are stored in granules inside the haemocytes (Destoumieux et al., 1997; Relf et al., 1999; Gross et al., 2001; Sotelo-Mundo et al., 2003; Srirachoen et al., 2005; Smith and Dyrnyda, 2015). Among crustaceans, freshwater crayfishes represent a diverse group of decapod crustaceans that play a central role in freshwater ecosystems. The same group has featured in various biological studies, involving disciplines such as physiology, ecology, immunology and neurobiology for over 130 years, since first being proposed as model organisms (Huxley, 1880; Crandall and De Grave, 2017; Cerenius et al., 2018).

The global distribution of freshwater crayfish is mainly limited to temperate latitudes and native crayfish species are absent in continental Africa and the Indian subcontinent (Scholtz, 2002). Crayfish live in different habitats, including permanent and seasonal rivers, streams and lakes, freshwater caves and springs, terrestrial burrows (Richman et al., 2015). Crayfish have been recognized as playing a significant role in determining ecosystem structure and function (Reynolds et al., 2013) and are also important economic species in Madagascar, Europe, China and the US State of Louisiana (Jones et al., 2006; Thies and Porche, 2007).

As reported in literature, several AMPs have been isolated from freshwater crayfish species, including such as *Pacifastacus leniusculus*, *Procambarus clarkii* and *Cherax quadricarinatus* (Donpudsai et al., 2010; Yu et al., 2016; Liu et al., 2016), and these compounds show a variable amino acidic composition and length, a high specificity and effective biological activity. The objective of this paper is to provide an overview of AMPs isolated from freshwater crayfish, highlighting their biological activities and their potential application in the development of new drugs.

## 2. Antimicrobial peptides from freshwater crayfish

Several studies have focused on the characterization and function of different AMPs identified in freshwater crayfish, highlighting their defensive role against bacterial infections. Among the most studied AMPs reported in literature are: Anti-Lipopolysaccharide Factors (ALFs), crustins, lysozymes, astacidins, arasins and hemocyanins (precursors of antibacterial and antifungal peptides).

### 2.1. Anti-Lipopolysaccharide Factors (ALFs)

The first ALF was discovered in the American horseshoe crab *Limulus polyphemus* (Muta et al., 1987). ALFs are considered an important AMPs, playing a fundamental biological role in immune defence mechanisms and their expression differs in relation to viral, bacterial and fungal challenges (Liu et al., 2005; Zhang et al., 2010). They are described as a group of basic proteins that bind and neutralize lipopolysaccharide (LPS). These molecules have a signal peptide and a conserved LPS-binding domain (LPS-BD) which can bind and neutralize LPS activity by mediating the haemocyte degranulation and initiating the intracellular coagulation cascade (Morita et al., 1985). For the first time, an ALF form has been identified in *P. leniusculus*, being able to influence the replication of White spot syndrome virus (WSSV) (Liu et al., 2006). Currently, several forms of ALFs have already been identified in other freshwater crayfish (Table 1) in particular in the red swamp *P. clarkii*, where they are involved in the innate immune defence pathway against WSSV (Huang et al., 2017) and bacterial pathogens, including Gram-positive bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus megaterium*), Gram-negative bacteria (*Escherichia coli*, *Vibrio anguillarum*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and fungi (*Pichia pastoris* and *Candida albicans*) (Sun et al., 2011; Zhu et al., 2019). An ALF factor with a significant antimicrobial activity has also been isolated from *C. quadricarinatus* (*CqALF*) and was effective against both Gram-positive and Gram-negative bacteria, in particular against *S. aureus* and *Shigella flexneri* (Lin et al., 2016, Fig. 1).

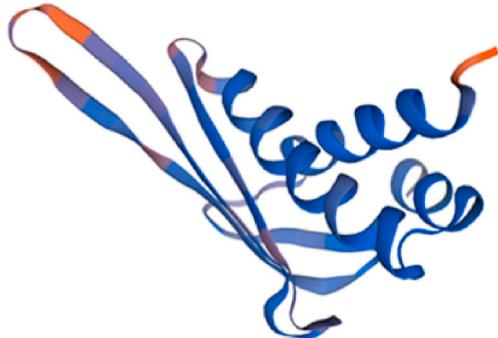
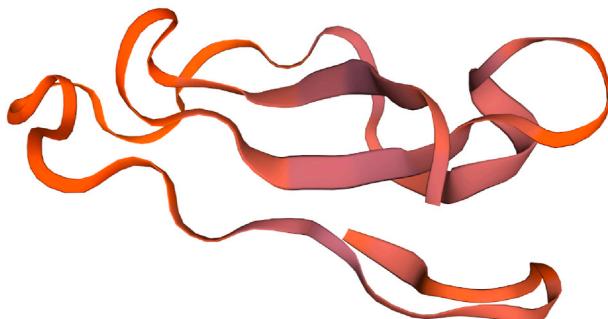
### 2.2. Crustins

Crustins are considered as the largest AMP family in crustaceans. They are defined as cationic cysteine-rich AMPs that show a signal sequence, variable regions at the N-terminus and a single whey acidic protein (WAP) domain with eight cysteine residues at the C-terminus (Smith et al., 2008). The signal sequence at the N-terminus comprises 16–24 amino acids, mainly represented by valine residues. The cleavage site which determines the end of the signal sequence is usually located between alanine and glycine, whilst in some crustins, this is situated between glycine and glutamine, alanine or threonine (Smith et al., 2008). It has been hypothesized that the same signal sequence may be involved in the *trans*-membrane transportation of proteins, as in insects and mammals, and the mature proteins or peptides lacking the signal sequence are released by exocytosis (Destoumieux et al., 2000; Muñoz et al., 2002; Smith et al., 2008). In contrast, the WAP domain is characterized by a four-disulfide core (4DSC) stabilized by eight conserved cysteine residues (Ranganathan et al., 1999). Generally, WAPs are small secretory proteins that show protease inhibitory properties and are

**Table 1**

Anti-lipopolysaccharide factors identified in freshwater crayfish.

Anti-lipopolysaccharide factors	Species	Accession number	Coded amino acid	Molecular weight (kDa)	References
PcALF	<i>P. clarkii</i>	MF185749	107 <sup>a</sup> /103 <sup>b</sup>	11.7 <sup>a</sup> /13.9 <sup>b</sup>	Zhu et al. (2019)
PcALF1	<i>P. clarkii</i>	MH538269	122 <sup>a</sup> /123 <sup>b</sup>	13.8 <sup>a</sup> /10.9 <sup>b</sup>	Sun et al. (2011)
CqALF	<i>C. quadricarinatus</i>	KX083340	123 <sup>a</sup> /123 <sup>b</sup>	13.3 <sup>a</sup> /13.3 <sup>b</sup>	Lin et al. (2016)
ALF	<i>P. leniusculus</i>	EF523760	120 <sup>a</sup>	13.4 <sup>a</sup>	Liu et al. (2006)

<sup>a</sup> Coded amino acid and molecular weight obtained using UniProt database (<https://www.uniprot.org/>).<sup>b</sup> Coded amino acid and molecular weight reported in references.**Fig. 1.** One examples of domain structures of Anti-Lipopolysaccharide Factors: CqALF of *C. quadricarinatus*. The structural models were created using Swiss-Model serve (<http://swissmodel.expasy.org/>).**Fig. 2.** One examples of domain structures of Crustin: Plcrustin 2 of *P. leniusculus*. The structural models were created using Swiss-Model serve (<http://swissmodel.expasy.org/>).

involved in growth and tissue differentiation; they sometimes can be expressed during cancer disease stages (Schalkwijk et al., 1999; Boucharad et al., 2006). It has been reported that the region between the signal sequence and the WAP domain is variable due to the different amino acid composition. Furthermore, crustins can be distinguished into three sub-families based on their domain architecture (Smith et al., 2008). Type I crustins contain a cysteine-rich region between the signal sequence and the WAP domain, represented by an incomplete 4DSC. This type of crustin can be found in lobster, crayfish and crab species (Stoss et al., 2003; Hauton et al., 2006; Brockton et al., 2007; Christie et al., 2007; Jiravanichpaisal et al., 2007; Liu et al., 2016). Type II crustins additionally possess a glycine-rich region followed by a cysteine-rich region and a WAP domain; these crustins occur frequently in shrimps (Smith et al., 2008; Liu et al., 2016). Type III crustins lack the typical motifs of both Type I and Type II, even though they contain a proline-arginine region at the front of the WAP domain (Smith et al., 2008). In last 20 years, several crustins have been described in crustaceans. The first crustin has been discovered in a shore crab *Carcinus maenas* and named carcinin (Schnapp et al., 1996).

Different types of crustins have been also identified in various

freshwater crayfish (Table 2). A type-I crustin has been isolated from *P. clarkii* that showed a wide antimicrobial activity against both Gram-positive and Gram-negative bacteria (*S. aureus*, *V. anguillarum*, *Aeromonas hydrophila*, *B. subtilis*, *Streptococcus agalactiae*, *Micrococcus luteus*, *E. coli* and *K. pneumoniae*) (Liu et al., 2016). Recently Du et al. (2019) have discovered a new isoform of crustin belonging to Type I, named as *Pc-crustin 4*, that inhibited the growth of both Gram-positive and Gram-negative bacteria (*S. aureus* and *Edwardsiella ictaluri*). A peculiar Type III crustin has also been identified in *P. clarkii*, named as *Pc-SWD* (Du et al., 2010), which belongs to the single WAP domain-containing peptides (SWDs). These peptides have already been detected in penaeid shrimps (Supungul et al., 2004; Amparyup et al., 2008; Jiménez-Vega et al., 2004; Rojinnakorn et al., 2002; Jia et al., 2008) and perform antimicrobial and inhibitory proteinase activities (Sallanave, 2000, 2002). SWDs containing-proteins have been clustered among crustin Type III molecules by Smith et al. (2008), due to the similarities with crustins, but they lack both the Type II molecules' Gly-rich domain and the Cys-rich region present in both Type I and Type II crustins (Smith et al., 2008). A novel Type-I crustin (*CqCrs*) has been characterized in the red claw crayfish *C. quadricarinatus*, showing binding and antibacterial activity in response to different bacteria (Yu et al., 2016), whereas two bacteria-induced genes, *Plcrustin1* and *Plcrustin2*, have been isolated from the haemocytes of *P. leniusculus* and these inhibited the growth of the Gram-positive bacterium *M. luteus* M1 11 when expressed (Donpudsa et al., 2010; Jiravanichpaisal et al., 2007) (see Fig. 2).

### 2.3. Lysozymes

Lysozymes are another important component of the innate immune system, being widely distributed in vertebrates, invertebrates, plants and microorganisms. These molecules show a broad spectrum of antimicrobial activities against bacteria, fungi and viruses and are also involved in several physiological processes such as digestion, inflammation, immune-modulation and anti-tumour pathways (Prager and Jolles, 1996; Regel et al., 1998; Guani-Guerra et al., 2010; Tassanakajon et al., 2013). Lysozymes show muramidase activity by hydrolyzing the β-1,4-glycosidic bonds of bacterial peptidoglycan and by killing both Gram-positive and Gram-negative bacteria (Chai et al., 2017). In the animal kingdom, principal lysozymes are classified into four groups, based on their differences in amino acid sequence, chemical properties and biological activities: chicken or conventional type lysozyme (c-type), goose-lysozyme (g-type), invertebrate-lysozyme (i-type) and chalaropsis type (ch-type) (Hancock and Scott, 2000; Callewaert and Michiels, 2010). The first two groups are characteristic of vertebrates and invertebrates, whereas i-types have been identified only in invertebrates. Several c-type lysozymes have been studied in marine crustaceans (Sotelo-Mundo et al., 2003; Bu et al., 2008; Mai and Hu, 2009; Ye et al., 2009; Kaizu et al., 2011; Karthik et al., 2014) while in freshwater crayfish, the knowledge about these molecules is still scarce. To date, lysozymes have been essentially characterized in *P. clarkii* (Table 3), in particular *Pclys1*, *Pclys-i3* (Fig. 3) and *PcLyzc*, have exhibited antimicrobial activity against both Gram-positive bacteria (*S. aureus*, *M. luteus*, *B. thuringiensis* and *B. subtilis*) and Gram-negative bacteria (*P. aeruginosa*, *A. hydrophila*, *V. anguillarum* and *E. coli*)

**Table 2**

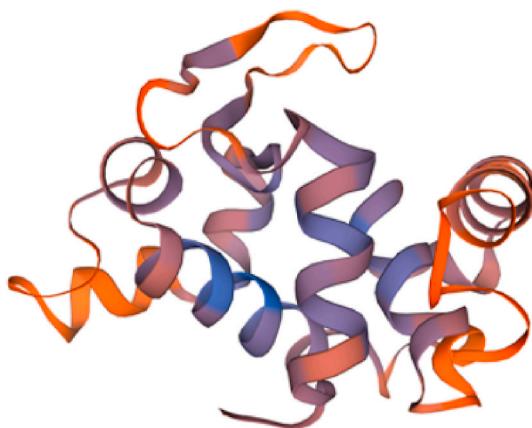
Crustins identified in freshwater crayfish.

Crustins	Species	Accession number	Coded amino acid	Molecular weight (kDa)	References
PcCru	<i>P. clarkii</i>	MF185748	110 <sup>a</sup> /110 <sup>b</sup>	12.3 <sup>a</sup> /12.3 <sup>b</sup>	Liu et al. (2016)
Pc-crustin 4	<i>P. clarkii</i>	–	122 <sup>b</sup>	11.7 <sup>b</sup>	Du et al. (2019)
CqCrs	<i>C. quadricarinatus</i>	KF773763	107 <sup>a</sup> /107 <sup>b</sup>	11.7 <sup>a</sup> /11.7 <sup>b</sup>	Yu et al. (2016)
Plcrustin 1	<i>P. leniusculus</i>	ABP88042	118 <sup>a</sup> /102 <sup>b</sup>	13.4 <sup>a</sup> /11.7 <sup>b</sup>	Donpudsaa et al. (2010)
Plcrustin 2	<i>P. leniusculus</i>	ABP88043	109 <sup>a</sup> /93 <sup>b</sup>	12.3 <sup>a</sup> /10.6 <sup>b</sup>	Donpudsaa et al. (2010)
Plcrustin 3	<i>P. leniusculus</i>	ABP88044	157 <sup>a</sup>	16.5 <sup>a</sup>	Jiravanichpaisal et al. (2007)

<sup>a</sup> Coded amino acid and molecular weight obtained using UniProt database (<https://www.uniprot.org/>).<sup>b</sup> Coded amino acid and molecular weight reported in references.**Table 3**

Lysozymes identified in freshwater crayfish species.

Lysozymes	Species	Accession number	Coded amino acid	Molecular weight (kDa)	References
Pclys 1	<i>P. clarkii</i>	AXR98475	152 <sup>a</sup> 131 <sup>b</sup>	16.51 <sup>a</sup> 14.1 <sup>b</sup>	Zhang et al. (2010)
PcLys-i3	<i>P. clarkii</i>	MG910500	157 <sup>a</sup> 137 <sup>b</sup>	16.7 <sup>a</sup>	Liu et al. (2018)
PcLyzc	<i>P. clarkii</i>	MG921601	144 <sup>a</sup> 144 <sup>b</sup>	16.5 <sup>a</sup> 14.3 <sup>b</sup>	Liao et al. (2018)

<sup>a</sup> Coded amino acid and molecular weight obtained using UniProt database (<https://www.uniprot.org/>).<sup>b</sup> Coded amino acid and molecular weight reported in references.**Fig. 3.** One example of domain structures of Lysozymes: *PcLys-i3* of *P. clarkii*. The structural models were created using Swiss-Model serve (<http://swissmodel.expasy.org/>).

(Zhang et al., 2010; Liu et al., 2018; Liao et al., 2018). However, the characterization of this antimicrobial molecules new group of AMPs may contribute to a broader understanding of innate immune response in freshwater crayfish.

#### 2.4. Arasins

Arasins represent a peculiar group of AMPs. In marine crustaceans, an arasin-like peptide has been isolated from the mud crab *Scylla paramamosain* and it showed antimicrobial activity against both Gram-positive and Gram-negative bacteria (Imjongjirak et al., 2011). They have mainly been found in decapod crustaceans such as *H. araneus* (Stensvåg et al., 2008) and in freshwater crayfish, arasins have been poorly investigated. Regarding freshwater crustacean only two antimicrobial peptides with a similar sequence to the arasins isolated from the decapod crustacean *H. araneus* have been identified in the red swamp crayfish *P. clarkii*, being named as *Pc-arasin1* and *Pc-arasin2* (Table 4). The *Pc-arasins* are cationic AMPs and exhibit broad antimicrobial

**Table 4**

Arasins identified in freshwater crayfish species.

Arasins	Species	Accession number	Coded amino acid	Molecular weight (kDa)	References
<i>Pc-</i> <i>arasin</i> 1	<i>P. clarkii</i>	–	42 <sup>a</sup>	4.6 <sup>a</sup>	Chai et al. (2017)
<i>Pc-</i> <i>arasin</i> 2	<i>P. clarkii</i>	–	50 <sup>a</sup>	5.5 <sup>a</sup>	Chai et al. (2017)

<sup>a</sup> Coded amino acid and molecular weight reported in references.

properties towards three Gram-positive bacteria (*S. aureus*, *M. luteus* and *B. subtilis*) and three Gram-negative bacteria (*A. hydrophila*, *E. coli* and *V. anguillarum*) (Chai et al., 2017). It has been demonstrated that this antimicrobial activity is analogous to the one exhibited by arasin 1 and arasin-like *Sp* isolated from *H. araneus* and *S. paramamosain* (Stensvåg et al., 2008; Imjongjirak et al., 2011).

#### 2.5. Astacidins

Astacidins are considered a peculiar group of proline-arginine rich peptides. Several astacidins were isolated in crayfishes (Table 5). The first astacidin, a peptide with 16 amino acid residues named as astacidin 1, has been isolated from the plasma of *P. leniusculus* and has been tested against Gram-positive (*B. megaterium*, *B. subtilis*, *S. aureus* Cowan 1, *S. aureus* JC-1, *M. luteus*) and Gram-negative (*S. flexneri*, *P. vulgaris*, *E. coli*, *P. aeruginosa*) bacteria, showing an inhibition of bacterial growth (Lee et al., 2003). Moreover, synthetic peptides (*SP-1*, *SP-2*, *SP-3*, and *SP-4*) have been obtained from the authentic astacidin 1 that showed low antibacterial activity. Among these synthetic peptides, synthetic astacidin 1 (*SP-1*) has showed similar antibacterial activity as the authentic astacidin against Gram-positive bacteria such as *B. megaterium*, *B. subtilis*, *M. luteus* whereas it had lower antimicrobial activity against Gram-negative bacteria. In contrast, the amino-terminal truncated peptides (*SP-2*, *SP-3* and *SP-4*) have demonstrated lower antibacterial activity than *SP-1*. However, it could be argued that the different antibacterial activity between the authentic astacidin 1 and the synthetic astacidin 1 could be linked to the different solubility of the peptides in water: synthetic peptide is less soluble than authentic peptide (Lee et al., 2003). Another peptide of 14 amino acid residues proline/arginine rich, designated as astacidin 2, has been isolated from the haemolymph of the freshwater crayfish *P. leniusculus* and has showed broad antibacterial activity against Gram-positive (*S. aureus*, *B. megaterium*, *B. subtilis*) and Gram-negative bacteria (*S. flexneri*, *P. vulgaris*, *E. coli*, *P. aeruginosa*). In particular, astacidin 2 has been synthesized as a prepropeptide and is apparently processed at the N-terminus and C-terminus by two proteinases (Jiravanichpaisal et al., 2007). Similar antibacterial activity of astacidin has also been found in *P. clarkii*. In fact, Shi et al. (2014) have isolated an antibacterial peptide designated as *PcAst* that showed high identity with astacidin 2 isolated from the crayfish *P. leniusculus* by Jiravanichpaisal et al. (2007). In

**Table 5**

Astacidins identified in freshwater crayfish species.

Astacidins	Species	Accession number	Coded amino acid	Molecular weight (kDa)	References
Astacidin 1	<i>P. leniusculus</i>	–	16 <sup>b</sup>	1.9 <sup>b</sup>	Lee et al. (2003)
Astacidin 2	<i>P. leniusculus</i>	ABH05920	42 <sup>a</sup> 14 <sup>b</sup>	4.7 <sup>a</sup> 1.8 <sup>b</sup>	Jiravanichpaisal et al. (2007)
PcAst	<i>P. clarkii</i>	GQ301199	43 <sup>a</sup> 20 <sup>b</sup>	4.7 <sup>a</sup> 2.3 <sup>b</sup>	Shi et al. (2014)
PcAst -1a	<i>P. clarkii</i>	MN848347	43 <sup>a</sup> 20 <sup>b</sup>	4.7 <sup>a</sup>	Rončević et al. (2020)
PcAst -1b	<i>P. clarkii</i>	MN848348	48 <sup>a</sup> 25 <sup>b</sup>	5.4 <sup>a</sup>	Rončević et al. (2020)
PcAst -1c	<i>P. clarkii</i>	MN848349	48 <sup>a</sup> 25 <sup>b</sup>	5.5 <sup>a</sup>	Rončević et al. (2020)
PcAst -2	<i>P. clarkii</i>	MN848350	53 <sup>a</sup> 31 <sup>b</sup>	6.2 <sup>a</sup>	Rončević et al. (2020)
PcAst -3	<i>P. clarkii</i>	MN848351	50 <sup>a</sup> 28 <sup>b</sup>	5.7 <sup>a</sup>	Rončević et al. (2020)

<sup>a</sup> Coded amino acid and molecular weight obtained using UniProt database (<https://www.uniprot.org/>).<sup>b</sup> Coded amino acid and molecular weight reported in references.

addition, the synthetic small peptide for *PcAst* (*SPcAst*) (Fig. 4) has revealed inhibitory activity against Gram-positive and Gram-negative bacteria tested whereas it showed no inhibitory activity against *B. subtilis* and *M. luteus* (Shi et al., 2014). It has also been demonstrated that *SPcAst* exhibited binding ability to bacterial cell wall components such as peptidoglycan, lipopolysaccharide and lipoteichoic acid. However, comparing the antibacterial activity of *SPcAst* and astacidin 2 from the freshwater crayfish *P. leniusculus*, in terms of inhibitory concentration, we could find some differences in their antimicrobial properties. For some bacteria such as *P. aeruginosa*, the inhibition concentration of *SPcAst* was lower than that of astacidin 2 whereas for other bacteria the inhibition concentration of *SPcAst* was much higher (Jiravanichpaisal et al., 2007; Shi et al., 2014). These differences could probably linked to the different origin of two peptides: astacidin 2 is a natural antibacterial peptide isolated from the haemolymph (Jiravanichpaisal et al., 2007) whereas *SPcAst* was a synthetic peptide that lacks post-translational modifications (Shi et al., 2014). Recently, four novel proline-rich antimicrobial peptides (PR-AMP) have been identified from the transcriptome of *P. clarkii* (Rončević et al., 2020). These peptides named as *PcAst-1a*, *PcAst-1b/c*, *PcAst-2* and *PcAst-3* are related with the previously identified hemocyte-specific PR-AMP astacidin 1 and are encoded by the multi-genic astacidin gene family. In particular, three synthetic *PcAst* peptides (*PcAst-1a-1b/1c*, *PcAst-2*) have showed a broad range of anti-bacterial properties against *S. aureus*, *E. coli* and *A. baumannii* (Rončević et al., 2020), in relation to the different medium broth: in particular, the study has demonstrated that the antimicrobial activity of *PcAst-1a-1b/1c*, *PcAst-2* was low (<32 μM) in full MH medium whereas in 20% MH broth it ranged from poor (≥32 μM for *PcAst-1a*) to selectively active (*PcAst-2* against *A. baumannii*) to potent with a broad spectrum against both Gram-positive and Gram-negative bacteria (*PcAst -1b/c*) (Rončević et al., 2020).



**Fig. 4.** One example of domain structures of Astacidins: *PcAst* of *P. clarkii*. The structural models were created using Swiss-Model serve (<http://swissmodel.ex.pasy.org/>).

### 3. Signalling pathways of AMP regulation in freshwater crayfish

The innate immune responses in mammals is based on specific molecular motifs known as pathogen-associated molecular patterns (PAMPs), including lipopolysaccharides (LPS), peptidoglycan (PGN), double-stranded RNA (dsRNA) and β-glucans (GLU) of microorganisms, which in turn are recognized by germ-line receptors called pattern recognition receptors (PRRs) (Janeway and Medzhitov, 2002). In invertebrates, including freshwater crustaceans, two main signalling pathways are included in innate immunity against invading pathogens, regulating the expression of AMPs: Toll and Immune Deficiency (IMD) pathways.

#### 3.1. Toll pathway

Toll or Toll-like receptors (TLRs) are considered the most extensively studied PRRs due to their central role in pathogen recognition and activation of the innate immunity (Akira et al., 2006). The first Toll receptor was initially identified in *Drosophila melanogaster* (named dToll) and its role in the dorsal-ventral embryonic development was demonstrated (Anderson and Nüsslein-Volhard, 1983). Toll/TLRs are defined as Type I integral membrane glycoproteins as characterized by their domain organization. Tolls and TLRs show an ectodomain that includes leucine-rich repeats (LRRs), a transmembrane domain and an intracellular Toll/interleukin-1 receptor (TIR) domain (Bowie and O'Neill, 2000). This mechanism starts with the activation of a proteolytic cascade that cleaves pro-Spätzle to Spätzle. It has been reported that Spätzle is proteolytically activated by a serine protease, but it is still unknown how this ligand is activated (Sánchez-Paz and Muhlia-Almazán, 2020). Spätzle acts as a ligand for the cell membrane Toll receptor, leading to an immune signal transduction and, consequently, to the production of AMPs (Shi et al., 2009). In particular, a trimeric complex composed by MyD88/Tube/Pelle is formed, which recruits TRAF6 to form a receptor complex, and in parallel Pellino, a highly conserved component of the Toll pathway, which binds phosphorylated Pelle/IRAK (Grosshans et al., 1999), increases the activity of Dorsal (Sánchez-Paz and Muhlia-Almazán, 2020). After the formation of this complex, Pelle is auto-phosphorylated and dissociated from the complex Dorsal/Cactus (Li and Xiang, 2013). The Dorsal-related Immunity Factor (DIF) has been discovered firstly in *Drosophila* (Ip et al., 1993) and regarding shrimps reviewed by Li and Xiang (2013). However, its participation in binding to the Dorsal/Cactus complex and in its translocation into the nucleus has not been investigated yet in crustaceans (Sánchez-Paz and Muhlia-Almazán, 2020). Moreover, in this group of arthropods the trimeric complex MyD88/Tube/Pelle binds to the activated Toll receptor (Wang et al., 2011; Zhang et al., 2012; Li and Xiang, 2013; Li et al., 2014), generating the transduction signal to the Dorsal/Cactus complex that regulates the Toll-dependent gene expression of AMPs and of a significant number of innate immune responsive genes (Sánchez-Paz and Muhlia-Almazán, 2020). With respect to freshwater crayfish, in *P. clarkii*, six Toll receptors (*PcToll*, *PcToll2*, *PcToll3*, *PcToll4*, *PcToll5* and *PcToll6*) have been described in previous

papers (Wang et al., 2015b; Lan et al., 2016a, b; Huang et al., 2017; 2018) and were highly expressed in the intestine, gills, haemocytes, heart and hepatopancreas of *P. clarkii* individuals. It has been reported that these key components of the invertebrate's innate immune system regulate the expression level of some AMPs in *P. clarkii*, such as crustins *Cru1* and *Cru2*, *ALF1*, *ALF2* and *ALF3*, and lysozyme 1 (*Lys1*), after exposure to *V. anguillarum* and *S. aureus*. In particular, *PcToll3* influences the expression of *PcMyd88*, tumour necrosis factor-associated factor 6 (PcTRAF6) and *PcDorsal*, which are similar of those of the *Drosophila* Toll signalling pathway (Lan et al., 2016b). Among the components of the Toll or Toll-like receptor signalling pathway, tumor necrosis factor receptor-associated factor 6 (TRAF6) is a multifunctional molecule, which is conserved from *Drosophila* to human (Mao et al., 2017). In the red swamp crayfish *P. clarkii*, *Pc-Traf6* was knocked down after exposure to *S. aureus* and *E. ictaluri* and the expression levels of some immunity effectors (ALF3, Lysozyme 1, Lectin 1 and Crustin 2) were significantly suppressed (Li et al., 2020). In the Chinese mitten crab *E. sinensis*, the myeloid differentiation factor 88 (My88), an essential adapter protein that is involved in the activation of the Toll-like receptor/interleukin-1 receptor mediated signalling pathway, has been identified and named as *EsMy88*. This factor can bind to the cytosolic adaptor *EsTube* and is involved in regulating the transcription of *ALF1* and *ALF2*, *Cru1* and *Cru2*, and *Lys* when exposed to *V. parahaemolyticus* (Huang et al., 2014). However, it has increasingly been demonstrated that the effector molecules induced after Toll pathway activation shows antimicrobial, antifungal and antiviral activity, regulating the expression of immune related genes (Huang et al., 2018) and unlike the Toll-to-Spätzle binding in *Drosophila*, shrimp Tolls have the ability to bind directly to the molecular patterns associated with pathogenic bacteria with Dorsal translocation in the nucleus to regulate the expression of different AMPs (Sun et al., 2017). Shrimp Tolls are pattern recognition receptors and the Toll pathway is different from the *Drosophila* Toll pathway but identical to the mammalian Toll-like receptor pathway (Sun et al., 2017).

#### 4. IMD pathway

Another important signalling pathway is the IMD pathway, which mediates the immunity against Gram-negative bacteria and which regulates the expression of AMPs genes in *Drosophila* (Gottar et al., 2002). This mechanism is based on the recognition of the meso-diaminopimelic acid-type peptidoglycan (PGN) from Gram-negative bacteria that binds to the PGRP-LC receptor complex (Leulier et al., 2003; Kaneko et al., 2004). The signal originated at the level of IMD is propagated through dTAK1 to Kenny (Key) and Ird5, which enhances the phosphorylation of Relish and, via dFADD (Drosophila Fas-associated death domain protein), the activation of caspase-8-homologue Dredd which is required for the cleavage of phosphorylated Relish (Lan et al., 2013). In *Drosophila*, the C-terminal part (Rel-49) remains in the cytoplasm whereas the N-terminal part (Rel-68) translocates into the nucleus, activating the transcription of genes for AMPs (Myllymäki and Rämet, 2014). The ubiquitination state (mediated by Iap2 and E2-ubiquitin-conjugating enzymes UEV1) is essential for Imd pathway-mediated AMP expression and for bacterial resistance (Rutschmann et al., 2000; Silverman et al., 2000; Lu et al., 2001; Kleino et al., 2005; Paquette et al., 2010; Meinander et al., 2012). IMD homologues have been observed for the first time in *P. clarkii* and named as *PcIMD* and *FcIMD* respectively (Lan et al., 2013). In particular, in *P. clarkii*, the pathway *PcIMD* regulated the expression of three kinds of AMP genes, including crustins, (*Cru 1* and *Cru 2*) *ALF 1*, *ALF 2* and *Lys* upon exposure to *V. anguillarum*. The IMD pathway has a conserved function for AMP regulation, within the crayfish immunity against Gram-negative bacteria (Lan et al., 2013; Liu et al., 2018). It has been reported that *E. sinensis* Relish is functional within the immune response against *Pichia methanolica* and *Listonella anguillarum* (Li et al., 2010) and two Relish isoforms, designated as *SpcRelish* and *LpcRelish*, have been isolated from *P. clarkii* and these can regulate the expression levels of different AMPs upon exposure to

*V. parahaemolyticus*. In particular, the expression levels of AMP genes (*ALF-41125*, *ALF- 42430*, *Crustin-41354* and *Crustin-42993*) were increased in *V. parahaemolyticus*-exposed *SpcRelish*-silenced crayfish, whereas the expression levels of *ALF-7032*, *ALF-9228*, *ALF-13162*, *ALF-42430*, *Crustin-41354*, *Crustin-42012* and *Crustin-42993* were down-regulated in *V. parahaemolyticus*-exposed *LpcRelish*-silenced crayfish (Zhang et al., 2020). Moreover, the caspase, an aspartate-specific cysteine protease, is considered an essential module in the IMD pathway, playing a central role in the process of apoptosis and in withstanding pathogen infection (Sahtout et al., 2001; Wongprasert et al., 2003, 2007; Wang et al., 2008; Flegel and Sritunyalucksana, 2011; Kim et al., 2014). A gene named *PcCaspase-3C* has been identified in *P. clarkii* and it regulated the expression of AMP genes in crayfish and related apoptotic activity. In particular, under infection with *S. aureus*, *V. anguillarum* or white spot syndrome virus (WSSV), the silencing of *PcCaspase-3C* significantly inhibited the expression level of some AMPs such as *ALF2*, *ALF5*, *ALF6*, *Lys*, *Cru3* and *Cru4* (Chen et al., 2019). Overall, few studies have investigated the role of other members of the IMD pathway in freshwater crayfish and their relationship with AMP expression. Thus, this regulatory mechanism has still to be fully elucidated.

#### 5. Potential applications of AMPs isolated from freshwater crayfish

Many studies reported in literature have highlighted the antimicrobial, antifungal and antiviral properties of AMPs discovered in various insects, including cecropins (Andrä et al., 2001; Arcidiacono et al., 2009; Guo et al., 2012, 2014; Hu et al., 2013; Liu et al., 2015; Lu et al., 2016; Wu et al., 2017; Zakharchenko et al., 2017) defensins (Bilikova et al., 2001), attacins (Hultmark et al., 1983; Kockum et al., 1984; Dushay et al., 2000; Geng et al., 2004) and drosocins (Bulet et al., 1993; Imler and Bulet, 2005; Gobbo et al., 2002). Some of these discovered insect-peptides has been applied in the formulation of hydrogels as cosmetic ingredients to deter dermatological pathogens (Pöppel et al., 2014, 2015; Rahnamaian and Vilcinskas, 2015) or in the therapy of chronic wounds (Eisenhardt et al., 2015). Moreover, other types of AMPs were isolated from frog skin, including ascaphins (Conlon et al., 2004), syphaxin (Dourado et al., 2007), brevinvin (Li et al., 2019), megins (Yang et al., 2016) and temporins (Malgieri et al., 2015). These AMPs could be considered therapeutic agents in anti-cancer treatments (Dong et al., 2020; Swithenbank et al., 2020; Wang et al., 2020; Zohrab et al., 2019). Although some studies have reported that AMPs isolated from *C. quadricarinatus* and *P. clarkii* could be used as prophylactic agents and to control infectious diseases in crustacean farming (Zhang et al., 2010; Li et al., 2008; Lin et al., 2016; Liao et al., 2018; Chai et al., 2017) to date, their use in medicine and aquaculture has not yet investigated. Thus, it could be argued that the potential therapeutic applications of freshwater crayfish AMPs should be further investigated in order to advance the current state-of-the-art concerning their potential application in both the aquaculture and medical fields.

#### 6. Concluding remarks

Freshwater crayfish lack adaptive immunity and thus their survival hinges on the production of a broad-spectrum of AMPs that allows them to develop a powerful defence mechanism to withstand infections. In fact, due to the variety of environments in which they live, freshwater crayfish have developed many responses within innate immunity. For this reason, they represent an important source of natural bioactive compounds. Currently, several bacteria have showed multidrug resistance due to the misuse of antibiotics and some super antibiotic resistant bacteria have emerged, representing a serious threat to human health (Zhang et al., 2015). Most AMPs isolated in freshwater crayfish are cationic, positively charged and their positive charge enhances the binding to negatively charged microbial surfaces through electrostatic

interactions. Thanks to their antibacterial and antifungal activity and to their ability to be active against some viruses, freshwater crayfish AMPs might be considered useful in medical and aquaculture fields. However, various aspects related to their exact function and signalling regulation pathways should be studied in greater detail. In particular, more quantitative studies should be encouraged to assess aspects such as host toxicity, haemolytic activity, anticancer and immunomodulatory properties. It would also be interesting to bridge existing related knowledge gaps, such as those concerning the role of AMPs in contrasting the formation of pathogenic bacterial biofilms, their possible application in extending the shelf-life of food and their co-synergic effects with other AMPs and conventional antibiotics. In addition, other components of the signalling pathways Toll and IMD should be taken into consideration in order to better understand their role in regulating AMP expression in freshwater crayfish. Therefore, considering their broad antimicrobial spectrum and the relative ease with which they can be managed in a laboratory environment, freshwater crayfish AMPs represent interesting candidates for therapeutic applications and for the development of new potential drugs.

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