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1 Type of the Paper (Review)

- 2 pH dependent antimicrobial peptides and proteins,
- 3 their mechanisms of action and potential as

4 therapeutic agents

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Academic Editor:12

13 Abstract: Antimicrobial peptides (AMPs) are potent antibiotics of the innate immune system that 14 have been extensively investigated as a potential solution to the global problem of infectious 15 diseases caused by pathogenic microbes. A group of AMPs that are increasingly being reported are 16 those that utilise pH dependent antimicrobial mechanisms and here, we review research into this 17 area. This review shows that these antimicrobial molecules are produced by a diverse spectrum of 18 creatures, including vertebrates and invertebrates, and are primarily cationic although a number of 19 anionic examples are known. Some of these molecules exhibit high pH optima for their 20 antimicrobial activity but in most cases, these AMPs show activity against microbes that present 21 low pH optima, which reflects the acidic pH generally found at their sites of action, particularly the 22 skin. The modes of action used by these molecules are based on a number of major structure / 23 function relationships, which include metal ion binding, changes to net charge and conformational 24 plasticity, and primarily involve the protonation of histidine, aspartic acid and glutamic acid 25 residues at low pH. The pH dependent activity of pore forming antimicrobial proteins involves 26 mechanisms that generally differ fundamentally to those used by pH dependent AMPs, which can 27 be described by the carpet, toroidal pore and barrel-stave pore models of membrane interaction. A 28 number of pH dependent AMPs and antimicrobial proteins have been developed for medical 29 purposes and have successfully completed clinical trials, including kappacins, LL-37, histatins and 30 lactoferrin, along with a number of their derivatives. Major examples of the therapeutic application 31 of these antimicrobial molecules include wound healing as well as the treatment of multiple cancers 32 and infections due to viruses, bacteria and fungi. In general, these applications involve topical 33 administration, such as the use of mouth washes, cream formulations and hydrogel delivery 34 systems. Nonetheless, many pH dependent AMPs and antimicrobial proteins have yet to be fully 35 characterized and these molecules, as a whole, represent an untapped source of novel biologically 36 active agents that could aid fulfillment of the urgent need for alternatives to conventional antibiotics, 37 helping to avert a return to the pre-antibiotic era.

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- 41 **PACS:** J0101
- 42
- 43 1. Introduction

Keywords: antimicrobial peptides and proteins; pH dependent antimicrobial activity; invertebrates;
 vertebrates;

44 A multiplicity of synergistic factors, including diminished pharmaceutical investment, clinical 45 over-prescription and misuse by the food industry has led to the increasing occurrence of microbial 46 pathogens with multiple drug resistance (MDR) and rendered infectious diseases the leading cause 47 of global mortality [1]. This bleak situation led the World Health Organization (WHO) to recently 48 predict that the uncurbed rise of MDR pathogens could see conditions in the 21st Century return to 49 those of the pre-antibiotic era when no antimicrobials were available for the treatment of many 50 common diseases [2]. In response, a major analysis by the WHO and a report from the O'Neill review, 51 sponsored by the UK Government, have concluded that the problem of antimicrobial drug resistance 52 can only be fully addressed by a coordinated global approach that operates through a number of 53 major interventions (Table 1) [3]. In particular, intervention six (Table 1) proposed the urgent 54 development of novel products and strategies that could provide alternatives to conventional 55 antibiotics, which has generated intensive research into antimicrobial design [4]. Examples of this 56 research range from revisiting old anti-infective strategies, such as phage therapy, which was popular 57 in Eastern European countries in the early 20th Century [5], to recently reported antimicrobial 58 strategies, such as the development of compounds whose antibiotic activity can be regulated by light 59 and sound [4,6-8]. One particularly promising approach proposed by O'Neill [3] was the therapeutic 60 development of antimicrobial peptides (AMPs), which are potent antibiotics of the innate immune 61 system [9,10]. The activity of these peptides against microbes involves relatively non-specific modes 62 of action at multiple sites with the result that microbial resistance to AMPs has a low incidence and 63 is generally due to inherent rather than adaptive mechanisms [11]. Based on these observations, the 64 generally held view is that microbial resistance to AMPs is unlikely to approach those of conventional 65 antibiotics, endowing these peptides with a major medical advantage [10], and currently, a number 66 of AMPs are in clinical trials (Table 2).

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Table 1: Major areas of intervention to combat antimicrobial drug resistance

1.	A global public awareness campaign.
2.	Improve sanitation and hygiene to prevent the spread of infection.
3.	Reduce the unnecessary use of antimicrobials in agriculture and their dissemination
	in the environment.
4.	Improve the global surveillance of drug resistance and antimicrobial consumption in
	humans and animals.
5.	Promote new rapid diagnostics to reduce use of unnecessary antimicrobials.
6.	Promote the development and use of vaccines and alternatives
7.	Improve the number, pay and recognition of people working in infectious disease.
8.	A global innovation fund for early stage and non-commercial research and
	development.
9.	Better incentives to promote investment for new drugs.

Table 1 was derived from [3]

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Table 2: Major examples of AMPs in clinical trials or in development

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Table 2 was derived from [12-15]

71 72

73 In order to develop AMPs as medically relevant anti-infective agents, there have been numerous 74 investigations into their antimicrobial mechanisms, which to date has shown that membrane 75 interaction is a requirement for virtually all of these mechanisms [9,10,16,17]. These investigations 76 have also shown that there are a number of major drivers in the membrane interactions of AMPs of 77 which the most important are charge, hydrophobicity and amphiphilicity [9,18,19]. The vast majority 78 of AMPs are cationic to help facilitate the targeting of microbes through direct electrostatic interaction 79 with anionic components of their membranes [19,20]. Nearly all AMPs are also amphiphilic, which 80 generates hydrophobic surfaces that are able to drive the partitioning of these peptides into microbial 81 membranes and hydrophilic surfaces that are able to stabilize these hydrophobic interactions via 82 electrostatic associations with the head group regions of these membranes [21,22]. Based on these 83 investigations, a variety of models have been proposed to describe the antimicrobial action of AMPs 84 with those most frequently reported appearing to be variants of the barrel stave pore and carpet type 85 mechanisms, which involve membrane disruption via discrete channel formation and non-specific 86 solubilisation respectively [23].

87 There have been many advances in understanding the mode of action used by AMPs but 88 although a number of earlier studies showed that pH can modulate the antimicrobial activity of these 89 peptides, no major review of this area of research appears to have been presented in the literature 90 [24-28]. However, it is now becoming increasingly clear that pH is a major driver in the membrane 91 interactions and biological activity of not only many AMPs but also a number of antimicrobial 92 proteins produced by eukaryotes (Table 3). To update on these antimicrobial molecules, here, we 93 present an overview of recent progress in the understanding of their modes of action along with the 94 development of their therapeutic and biotechnological potential.

95

96 2. An overview of pH dependent peptides and proteins with antimicrobial activity

97 In the 1980s and 1990s, a series of seminal studies, including work on the African clawed frog, 98 Xenopus laevis, and a number of mammals, led to what many take to be the first major description of 99 eukaryotic AMPs such as magainins, defensins and SAAPs [29]. However, in 1956, phagocytin from 100 humans, rabbits, horses and guinea pigs was reported to exhibit non-membranolytic activity against 101 a range of Gram-positive and Gram-negative bacteria that was enhanced by low pH [24,25]. The 102 peptide was not characterised or further investigated and today, it is not even known as to whether 103 phagocytin was rediscovered later and given an alternative name [30]. However, it would appear to 104 be a matter of historical fact that what was most likely the first AMP to be reported from eukaryotes 105 showed a pH dependent mode of action [24,25]. Since these earlier studies, it is now known that these 106 peptides are produced by virtually all multicellular organisms [31,32] and that an increasing number 107 of these molecules possess pH-dependent activity (Table 3).

108

109

Vertebrates	AMPs	Host organism	Key references
Fish	Gaduscidin-1 and	Gadus morhua	[33,34]
	gaduscidin-2		
Amphibians	Chensinin-1	Rana chensinensis	[35,36]
	Esculentin-2EM	Glandirana emeljanovi	This work
	Dermaseptin PD-3-7	Pachymedusa dacnicolor	[37]
Humans	Phagocytin		[24,25]
	Psoriasin		[38-40]
	β-microseminoprotein		[41].
	LL-37		[42]
	Hep-25 and hep-20		[43-48]
	Histatins		[49,50]
	Lactoferrin		[51]
	DCD-1(L)		[52-54]
	Kappacin A and kappacin B		[55,56]
Rabbits	Phagocytin		[24,25]
	Platelet microbiocidal		[57]
	proteins		
	NP1 and NP2		[58,59]
Horses	Phagocytin		[24,25]
Guinea pigs	Phagocytin		[24,25]
Mice	CRAMP		[42]
Cattle	Lactoferricin B		[60,61].
Invertebrates	AMPs	Host organism	Key references
Marine	Myticin C	Mytilus galloprovincialis	[62,63]
	KPS-1	Atrina pectinate	[64]
	Ci-PAP-A22 and Ci-MAM-	Ciona intestinalis	[65-68]
	A24		
	Clavaspirin and clavanins	Styela clava	[69-80]
	Styelins	Styela clava	[72,73,81]
Terrestrial	Hebraein	Amblyomma hebraeum	[82]
	Amoebapores	Entamoeba histolytica	[83-87]
	Acanthaporin	Acanthamoeba culbertsoni	[88]
	Caenopores	Caenorhabditis elegans	[89-93]

Table 3. AMPs with pH dependent activity.

110

111 2.1. Fish

112 Gaduscidin-1 (gad-1) and gaduscidin-2 (gad-2) were AMPs identified in the Atlantic cod, Gadus 113 morhua and shown to be highly, constitutively expressed in immune-relevant tissues [94,95]. Low pH 114 was found to enhance the activity of both peptides against Escherichia coli, which appeared to involve 115 membrane interaction, but interestingly, although gad-1 and gad-2 were predominantly α -helical at 116 neutral pH, acid conditions led to a large decrease in the levels of α -helicity possessed by these 117 peptides [33]. These results contrast with most α -helical AMPs where an enhanced capacity for 118 membranolysis and antimicrobial activity is generally associated with increased levels of this 119 secondary structure [96,97]. It was proposed that gad-1 and gad-2 each possessed a structural 120 plasticity, which facilitated an appropriate balance between amphiphilic and mixed hydrophobic / 121 hydrophilic structural features that promoted maximal levels of membrane interaction and 122 antibacterial activity [33]. Gad-1 and gad-2 were found to be histidine rich AMPs and the enhanced 123 capacity of these peptides for membranolysis and antimicrobial activity at low pH appeared to

124 involve these residues [33]. It is well established that histidine (pKa 6.5) is uncharged at physiological 125 pH but fully positively charged at low pH, thereby enhancing the potential of AMPs for interaction

125 pH but fully positively charged at low pH, thereby enhancing the potential of AMPs for interaction 126 with anionic membranes under acid conditions [50,98]. However, there also appeared to be a complex

relationship between the level of histidine residues possessed by these AMPs and their pH dependent

128 capacity for membrane interaction [33,34]. In response, molecular dynamic simulation studies were

129 undertaken and predicted that the number of sequential histidine pairs contained by gad-1 and gad-

130 2 were important to their ability for membrane disruption [34]. These AMPs possess one and two of

these histidine pairs respectively [94] and the N-terminal regions of both peptides, which included

this motif, were preferentially located proximal to membrane channels with which gad-1 and gad-2

133 were associated [34]. Based on the topology of the peptide-lipid interactions mediating the formation

134 of these channels, it was suggested that the antimicrobial action of gad-1 and gad-2 may involve the

135 use of a disordered toroidal pore type mechanism of membrane disruption [34,99,100].

136 2.2. Amphibians

137 Chensinin-1 is a histidine rich peptide produced by the frog, Rana chensinensis and recent 138 studies showed that low pH enhanced the positive charge of the peptide [35] and thereby, its ability 139 to kill Gram-positive bacteria, such as Bacillus cereus [36]. This antibacterial activity appeared to 140 involve the adoption of an extended structure, similar to that of other AMPs that are rich in specific 141 residues [101,102], which induced lysis of the B. cereus membrane [36]. Interestingly, the peptide 142 showed no activity against Gram-negative bacteria [36,103], which appeared to involve high affinity 143 binding between chensinin-1 and lipopolysaccharide (LPS) in the outer membrane of these bacteria 144 [103]. Maximin H5 from the toad, Bombina maxima, was also recently found to be ineffective against 145 Gram-negative bacteria due to high affinity binding to phosphatidylethanolamine (PE) in the 146 cytoplasmic membrane (CM) of these organisms [104,105]. Similar PE mediated mechanisms have 147 been proposed to mediate the resistance of microbes to other AMPs [104,105], supporting the growing 148 view that receptors could play a variety of roles in the biological activities of these peptides [23,98,106-149 108]. Esculentin-2EM (E2EM, previously gaegurin 4) is an α -helical peptide isolated from the frog, 150 Glandirana emeljanovi (formerly Rana rugosa) KIM [109,110], that is able to kill protozoa, fungi, Gram-151 positive bacteria and Gram-negative bacteria [110-112]. E2EM possesses a C-terminal cyclic region 152 stabilized by a disulphide bond (Rana box) that is conserved across many ranid AMPs and helps 153 stabilise pore formation by the peptide thereby promoting its antimicrobial action (Figure 154 1)[109,110,112-115]. Several models have been proposed to represent pore formation by E2EM and 155 the best supported by experimental evidence appear to be the toroidal pore and barrel stave 156 mechanisms (Figure 1). Here we present data showing that the linear reduced form of E2EM (E2EM-157 lin) possesses antimicrobial activity consistent with recent studies showing that the reduction of 158 cysteine-stabilized AMPs to generate peptides with novel mechanisms of antimicrobial activity may 159 form part of some innate immune systems [116,117]. Our results showed that E2EM-lin was active 160 against both Gram-positive and Gram-negative bacteria and appeared to exhibit pH dependent 161 antimicrobial activity, which parallels the molluscan cysteine stabilized AMPs, myticins, whose 162 reduced forms were described above to show pH dependent antibacterial and antiviral action [62,63]. 163 It was found that under the low pH conditions associated with the skin of frogs [118], E2EM-lin had 164 a general ability to induce the lysis of both anionic and zwitterionic membranes, which was enhanced 165 at higher pH (Table 4). These data clearly suggest that the C-terminal disulphide bond in the E2EM 166 Rana box region does not play a major role in its ability for membrane pore formation (Figure 1), 167 which supports earlier work [114,115]. In the case of DMPG, which is a key component of membranes 168 within Gram-positive bacteria [119], E2EM-lin induced relatively low levels of membrane lysis at acid 169 pH (< 25%). However, a shift to alkaline pH led to a large increase in the lytic activity of the peptide 170 to circa 95%, which was accompanied by a correspondingly large increase in its α -helical content of 171 circa 25% to give levels approaching 75% (Table 4). Previous studies have shown that E2EM-lin and 172 E2EM adopt highly similar α -helical structures in membranes and undergo oligomerization to form

173 pores [115,120]. Based on these data, we speculate that high pH may enhance the ability of E2EM-lin 174 to lyse DMPG membranes by increasing the potential of the peptide for pore formation through an 175 increased capacity for self-association. The segments of E2EM-lin involved in pore formation are 176 strongly amphiphilic α -helices with wide hydrophobic faces that would be maximized by alkaline 177 pH, promoting the potential for the mutual interactions involved in the formation of multimeric 178 species (Figure 1). In the case of DMPE, which is often taken to represent the membranes of Gram-179 negative bacteria [119], E2EM-lin induced high levels of membrane lysis at acid pH (60%). A move to 180 alkaline pH led to a relatively low increase in the lytic activity of the peptide, which was around 25% 181 and was accompanied by a decrease in its α -helical content of 20% to give levels of circa 30% (Table 182 4). These data would seem to indicate that E2EM-lin uses a different mechanism of lysis in the case 183 of DMPE membranes and it has previously been suggested that the peptide may adopt a number of 184 lipid interactive forms [115]. The functional significance of our data is not fully clear but given the 185 very high levels of membrane lysis induced by E2EM-lin at higher pH for each lipid investigated, 186 biological relevance is suggested, which forms the basis of ongoing investigations.

187

Lipid	рН	Lysis (%)	α -helicity (%)
DMPS	6	17	30
	8	63	49
DMPG	6	23	51
	8	94	73
DMPC	6	52	45
	8	73	15
DMPE	6	60	49
	8	83	31

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Table 4. The α -helical content and lysis levels of E2EM-lin

189 The levels of lysis exhibited by E2EM-lin were determined using a calcein release assay and the levels of α -

190 helicity shown by the peptide were measured using CD spectroscopy, all as previously described [105].

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200 Figure 1 was revised from [115] and Figure 1A shows models for pore formation by E2EM, which are 201 the toroidal pore and barrel stave mechanisms (Table 1) and are the best supported experimentally. 202 In both models, the N-terminal 23 residues of the peptide spans the bilayer and a glycine kink 203 orientates the 7 residue, C-terminal Rana box region of E2EM to lie parallel to the membrane surface. 204 In this orientation, the Rana box region of the peptide, which is a cystein stabilized macrocyclic 205 structure, interacts with the lipid head-group region of the membrane and stabilises pore formation 206 by E2EM [115]. The major difference between these models is that in the toroidal pore mechanism, 207 the membrane leaflets deform to allow the lipid head-group region to remain in contact with the 208 hydrophilic face of the E2EM membrane spanning region, which is not observed in the barrel stave 209 mechanism [23]. For clarity, two monomers of E2EM are shown in the schematic pore above but 210 oligomers formed by between five and ten peptide molecules have been proposed [115,120]. Similar 211 models of membrane interaction appear to apply to the linear reduced form of the peptide [115], 212 which is represented in our studies as E2EM-lin. Figures 1B and 1C show two-dimensional axial 213 projections [121] for the membrane spanning region and Rana box domain of E2EM, respectively, that 214 are involved in pore formation by the peptide. In both cases, these segments for amphipilic α -helices 215 with wide hydrophobic faces that our data suggest would be maximised by alkaline pH, thereby 216 promoting the potential for the mutual interaction of E2EM monomers and the formation of 217 multimeric species involved in pore formation.

218 In addition to cationic AMPS, anionic AMPs with pH dependent activity have also been reported 219 for amphibians, such as dermaseptin PD-3-7, which was isolated from the frog, Pachymedusa 220 dacnicolor [122]. In aqueous solution, the peptide showed an inherent propensity to adopt an extended 221 conformation and self-assemble into amyloid fibrils in a reversible pH-controlled manner [37]. At 222 low pH, dermaseptin PD-3-7 existed as amyloid-like β -sheet aggregates but at higher pH underwent 223 morphological changes, which led to the formation of metastable amorphous aggregates in a manner 224 that appeared to be mediated by deprotonation of the aspartic acid residues (pKa 3.9) and C-terminal 225 carboxyl groups possessed by the peptide. These amorphous aggregates induced damage to cells of 226 the insect, Spodoptera frugiperda, by an unidentified mechanism but showed no activity against E. coli 227 and Bacillus subtilis [37]. Based on these observations, it was suggested that amyloid formation by 228 dermaseptin PD-3-7 may act as a storage facility for the peptide similar to the depository function 229 proposed for the amyloidogenesis of pituitary peptide hormones [123]. Triggered by an increase in 230 pH, this storage facility would release a pre-formed, cytotoxic agent that contributed to the natural 231 defence strategy of the host amphibian [37]. However, it is worthy of note that the peptide was only 232 tested for activity against a small number of bacteria [37] and it is generally accepted that AMPs are 233 promiscuous in their antimicrobial mechanisms [29,124] with amyloid-mediated antibacterial 234 mechanisms increasingly being reported [23,125]. Interestingly, more recent studies on dermaseptin 235 PD-3-7 have shown that stereochemical modification of the peptide's second residue to form the 236 diastereomer [d-Leu2] strongly influenced the pH-triggered, morphological changes involved in 237 amyloid formation by dermaseptin PD-3-7, inducing a fundamental change in its superstructural 238 organization that was related to differences between the conformational propensities of these epimers 239 [126]. It was proposed by these latter authors that epimers of PD-3-7 may play a role as anionic AMPs, 240 or defence molecules, in the innate immune system of P. dacnicolor [126] and a similar proposal has 241 been made for the production of epimeric AMPs by other frogs and toads [9].

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243 2.3. Humans and other mammals

244 Psoriasin (S100A7) is a human, cystein stabilized α -helical protein [127] of the S100 family of 245 signalling proteins [128-130] that is known to have a role in the antimicrobial defence of the skin, 246 including serving as a multifunctional modulator of neutrophil activation [131,132]. The protein was 247 investigated for antimicrobial activity and shown to kill E. coli using pH independent mechanisms 248 that were primarily due to the depletion of Zn2+ [38,39] and more recently, the protein was identified 249 in vaginal fluid, appearing to help protect the female genital tract from infection [40]. Psoriasin was 250 also found to kill *Baciillus megaterium* but via the use of two different modes of action, which involved 251 Zn2+ depletion at neutral pH but membrane pore formation and oligomerisation of the protein at 252 low pH. This pore forming mechanism was not further investigated but evidence suggested that it 253 was likely to show some similarities to a barrel-stave pore type mode of action [38,39]. It is interesting 254 to note that psoriasin exhibits a number of structural and functional similarities to amoebapore A 255 (Figure 2) [39,133], which is a pH dependent antimicrobial protein from the protozoa, Entamoeba 256 histolytica that is discussed below [85]. In particular, psoriasin possesses a histidine residue in its C-257 terminal region [127] similar to amoebapore A [86] and based on these similarities, it can be 258 speculated that the enhanced action of psoriasin against B. megaterium at low pH may involve a 259 histidine mediated increased ability for pore formation and oligomerisation. β -microseminoprotein 260 (MSP), also named as PSP-94, is a human protein that is believed to have a protective role in prostate 261 carcinogenesis due to its ability to suppress the growth of tumours although more recent studies have 262 suggested that MSP may protect against prostate cancer by inhibiting fungal infection in this genital 263 region [134,135]. This suggestion was primarily based on recent work, which showed that the acid 264 conditions of the vagina promoted the ability of MSP in post coital seminal plasma to kill Candidia 265 albicans. This antifungal activity appeared to involve lysis of the organism's membranes and to be 266 mediated by a C-terminal fragment of MSP, which included a glutamic acid residue involved in the 267 ability of the protein to form coordinate bonds with Ca2+. It appeared that MSP coordination of Ca2+ 268 at neutral pH inhibited the antifungal activity of the protein but at low pH, electrostatic interaction 269 between the ion and the C-terminal glutamic acid of MSP (pKa 4.1) decreased, facilitating the ability 270 of the protein to kill C. albicans. Porcine MSP appeared to use a similar pH dependent antifungal 271 mechanism, suggesting that it may be a widespread innate immune factor active against C. albicans 272 and possibly helping to explain the low sexual transmission rate of vulvovaginal candidiasis in 273 humans [41]. C. albicans was also reported to be susceptible to the pH dependent activity of LL-37 274 and its derivatives, KS-30, and RK-31, along with CRAMP [42], which is a murine homologue of LL-275 37 [136]. It appeared that KS-30, and RK-31 were produced by the proteolytic cleavage of LL-37 in the 276 low pH environment of human sweat and that these pH conditions enhanced the ability all the 277 peptides tested to kill C. albicans via permeabilisation of the fungal membrane. The use of animal 278 models showed that although LL-37 and its derivatives were induced in murine skin in response to 279 *C. albicans* infection, this induction did not confer subcutaneous resistance to the organism [42]. Based 280 on these results, it was suggested that these peptides may be of primary importance in forming a 281 barrier against fungal infections on the skin surface [42], given that the dysregulated production of 282 LL-37 and its derivatives has been strongly associated with skin disease due to fungi and other 283 microbes [137-139]. Hepcidin, (hep-25) is a human β -sheet hormone that has a well-established role 284 in iron homeostasis [140] and has been shown to exhibit pH dependent antimicrobial activity [43]. In 285 particular, acid conditions been shown to enhance the activity of hep-25 and several of its isoforms, 286 such as hep-20, against the fungal pathogen, Candida glabrata [44], along with a range of Gram-287 negative bacteria, such as Pseudomonas aeruginosa, and Gram-positive bacteria, including 288 Enterococcus faecium [45,46]. Studies on E. coli suggested that the antibacterial action of these 289 peptides involved membranolytic mechanisms which were enhanced under acid conditions due to 290 the presence of histidine residues within their primary structure [47]. Based on these observations, it 291 was suggested that with acidic pH, the increase in positive charge of histidine residues in hep-25 and 292 hep-20 would promote their ability to target and lyse bacterial membranes, resulting in the death of 293 the host organism [43]. Hep-20 has also been shown to be effective against drug resistant C. glabrata 294 under the low pH and physiological conditions associated with the vagina, which led to the 295 suggestion that the peptide may form the basis of novel therapeutics for the control of vaginal 296 infections due to the organism [48]. Another group of human AMPs rich in histidine residues are the 297 histatins (hst), which are salivary peptides with antiviral, antibacterial and antifungal activity [141-298 146] and protecting the oral cavity from fungal pathogens appears to be to the primary role of these 299 peptides [147,148]. The pH of the oral cavity is mainly governed by that of saliva, which has a range 300 that generally varies between pH 5 and pH 8 [149,150] although significantly lower pH values can 301 occur on the surface of teeth due to the metabolic activities of cariogenic microorganisms [151], which 302 is able to promote the growth of fungi and form mixed species biofilms [152,153]. Low pH appears 303 able to enhance the antifungal activity of hst-1, hst-3 [154] and hst-5 [49], which, for example, 304 enhances the positive charge carried by hst-5 and facilitates its translocation into fungal cells to attack 305 intracellular targets [49]. However, a detailed description of the antifungal activity of these peptides 306 is lacking and a number of mechanisms have been proposed [141,143-146], such as complex formation 307 with iron to interfere with the cellular metabolism of the metal in fungi, such as *C. albicans* [155]. 308 Studies on human lactoferrin, which is a multifunctional iron-binding protein [156] found that low 309 pH enhanced the ability of sub-lethal levels of the protein to kill C. albicans, through multiple 310 mechanisms, including dissipation of the proton motive force (PMF) across the CM of the organism 311 [51]. This pH effect was attributed to increased electrostatic interactions between the peptide and 312 anionic components of the C. albicans membrane, thereby enhancing the ability of lactoferrin to 313 partition into fungal CM and generate lesions associated with PMF dissipation and the peptide's 314 antifungal action [51]. Sub lethal levels of lactoferrin have also been shown to be effective against 315 biofilms of P. aeruginosa [157] and the protein was found able to synergise the activity of other 316 antimicrobials against biofilms formed from P. aeruginosa and methicillin resistant Staphylococcus 317 aueus (MRSA) [158,159]. It was proposed that this anti-biofilm activity was due to the iron-binding

318 properties of the protein [157] but this does not appear to be the only mechanism involved in this 319 activity [160] and acidic pH is strongly associated with these sessile microbial communities [161]. 320 Lactoferricin B, which is a potent AMP derived from bovine lactoferrin [162], has also been shown to 321 possess pH dependent antimicrobial activity, killing bacteria and C. albicans at low pH via 322 mechanisms that appeared to involve membranolysis [60,61]. Low pH was also found to enhance the 323 activity of platelet microbiocidal proteins, which were isolated from leporine platelets and showed 324 activity against Staphylococcus aureus, E. coli, and C. albicans [57]. These results strongly supported the 325 mounting evidence that platelets serve important multiple roles in host defence against infection, 326 including the localized release of AMPs and other antimicrobial factors in response to microbial 327 colonization and other stimuli [163]. The pH dependent antimicrobial mechanisms of platelet 328 microbiocidal proteins were not further characterised but other studies showed that the antifungal 329 and antibacterial activity of a number of these AMPs involved dissipation of the PMF across the 330 cytoplasmic membrane of target organisms, which was able to synergise the activity of conventional 331 antibiotics [164]. These results parallel those described above for human lactoferrin, allowing the 332 speculation that low pH enhances the ability of some leporine platelet microbiocidal proteins to 333 interact with membranes of C. albicans and generate lesions associated with PMF dissipation and the 334 peptide's antifungal action. Other leporine AMPs shown to possess pH dependent activity are the 335 defensins, NP1 and NP2, which were found to permeabilise the outer membrane of *P. aeruginosa* most 336 efficiently at low pH although these peptides were ineffective against the organism under these pH 337 conditions. These AMPs are present in leporine macrophages and it was suggested that this pH 338 dependent membranolytic activity may synergise the antibacterial action of other defence molecules 339 under the acid conditions associated with phagocytosis [58,59].

340

341 **Figure 2.** Similarities between the structures of psoriasin and amoebapore A



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Figure 2 was revised from [39] and shows human psoriasin (**A**) and amoebapore A from the protozoa, *Entamoeba histolytica* (**B**). It can be clearly seen that these peptides show structural similarities and both have been shown to possess pH dependent mechanisms of antimicrobial activity that is enhanced by acid conditions [38-40,83-85,87]. In particular, psoriasin possesses a histidine residue in its C-terminal region [127] similarly to amoebapore A [86] and based on these similarities, it can be speculated that the enhanced antibacterial action of psoriasin at low pH may involve a histidine mediated increased ability for pore formation and oligomerisation. 350 A particularly important case of human AMPs with activity that can be influenced by pH is that 351 found in the airway surface liquid (ASL) of individuals with cystic fibrosis (CF) [165,166], which is a 352 lethal genetic disorder characterized by viscous mucus and bacterial colonization of the airways 353 [167]. In the mammalian respiratory system, the ASL represents a first line of pulmonary defence by 354 forming the interface between the environment and the host organism and helping to protect against 355 the action of inhaled and aspirated bacteria by producing a variety of antimicrobial molecules 356 [168,169]. These ASL molecules include AMPs, such as LL-37, HNP-1, HBD-1 and lactoferrin, along 357 with antimicrobial proteins, such as lysozyme, surfactant protein A and surfactant protein D 358 [165,166]. Many of these antimicrobial molecules also contribute to the pulmonary innate immune 359 system by adorning lattices of extracellular DNA, chromatin, enzymes and other proteins to form 360 neutrophil extracellular traps (NETs). These DNA complexes are released in response to the presence 361 of microbial pathogens and provide a mechanism for the localised concentration of effector 362 molecules. NETs have been reported able to eradicate microbial pathogens using a variety of 363 mechanisms, including the action of antimicrobial attachments and proteolytic degradation, as well 364 as neutralizing their activity by forming a physical barrier that prevents the dissemination of these 365 pathogens [166,170-172]. More recent studies have shown that the Human Short Palate Lung Nasal 366 Epithelial Clone 1 (SPLUNC1), which is a protein expressed in the upper airways of the lung, plays 367 multiple roles in pulmonary innate immunity. These roles include: the direct inhibition of bacterial 368 growth, the prevention of microbial biofilm formation and the regulation of other AMPs and 369 antimicrobial proteins, such as LL-37, HBD-2 and lysozyme [173-176]. However, in CF, mutations in 370 the cystic fibrosis transmembrane conductance regulator (CFTR) gene leads to reduced HCO3-371 secretion and produces an abnormally acidic pH in ASL [177,178], which studies on humans and 372 animal models have suggested can negatively affect the efficacy of ASL antimicrobial molecules and 373 predispose CF airways to microbial infection (Figure 3) [165,179]. For example, recent studies on a 374 porcine CF model showed that the low pH of the ASL inhibited the activity of LL-37, lactoferrin and 375 other AMPs when directed against S. aureus and P. aeruginosa [180,181], which are known, major CF 376 pathogens [182,183]. These acid conditions also reduced the ability of ASL AMPs to synergize their 377 activities when in combination with each other and with antimicrobial proteins, such as lysozyme 378 [180,181]. It has been further proposed that conditions of low pH in CF airways could reduce the 379 efficacy of AMPs and antimicrobial proteins that adorn NETs [170] along with the antimicrobial and 380 other biological activities of SPLUNC1 [179,184]. The mechanisms by which the low pH impairs the 381 activity of AMPs and antimicrobial proteins in CF airways are currently unclear but it has been 382 suggested that these mechanisms include a variety of contributions. For example, it has been 383 proposed that low pH in CF airways may mediate the degradation of AMPs via the activation of host 384 proteases, such as cysteine cathepsins, and microbial enzymes, such as aureolysin of S. aureus and 385 elastase of *P. aeruginosa*, and the immobilization of AMPs through binding to mucins, which are large, 386 anionic glycoproteins and the primary component of mucus. It has been further proposed that low 387 pH in CF airways may induce conformational changes in AMPs that reduce the ability of these 388 peptides to bind microbial membranes and cell wall components, such as lipid II 389 [165,166,170,180,181,185]. In addition to reducing the activity of AMPs, low pH appears able to 390 negatively impact on other defence factors of the ASL; for example, by increasing the rheological 391 properties of secreted mucins, decreasing ciliary beat frequency, impairing phagocyte function and 392 depleting ASL volume [165,166,179,185]. Based on these observations, it has been proposed that 393 connections between the loss of CFTR, reduced ASL pH, and impaired CF host defense function 394 could provide a paradigm for the identification of new therapeutic targets and strategies to reduce 395 the morbidity associated with CF lung disease [165].

396

397

Figure 3. The pH of airway surface liquid and the pathogenesis of cystic fibrosis





400 Figure 3 was revised from [165] and shows a scheme for how changes in ASL pH may influence the 401 pathogenesis of CF. In CF, the loss of CFTR function results in decreased HCO3⁻ conductance across airway 402 epithelial cells and leads to low pH in the ASL. Under these pH conditions, ASL AMPs, such as LL-37, 403 HNP-1, HBD-1 and lactoferrin, and antimicrobial proteins, such as lysozyme, surfactant protein A and 404 surfactant protein D, have reduced activity. Lower pH also leads to the increased viscosity of mucins, 405 decreased ciliary beat frequency, impaired phagocyte function and depleted ASL volume. These effects 406 lead to a decrease in the antimicrobial efficacy of the ASL and subsequently contribute to increased 407 respiratory infections in the CF airway, caused by both viral and bacterial pathogens [165,166,179,185].

408

409 A number of anionic AMPs with pH dependent activity have been identified in humans, 410 including DCD-1(L), which is proteolytically cleaved from dermcidin, which is also an anionic 411 peptide and found in human sweat [9,52,186,187]. DCD-1(L) is characterised by its broad range 412 antimicrobial activity, killing fungi, such as C. albicans, as well as Gram-positive bacteria, including 413 MRSA, Gram-negative bacteria, such as Salmonella typhimurium, and acid fast bacteria including 414 rifampin- and isoniazid-resistant Mycobacterium tuberculosis [188-193]. The peptide appears to exhibit 415 pH dependent antibacterial action [188] whereby low pH induces the peptide to adopt α -helical 416 structure on the bacterial membrane surface, leading to ion channel formation via Zn²⁺ stabilized 417 DCD-1(L) oligomers and death of the host organism through membrane disruption [52,53]. A more 418 recent study gives general support to this model and suggested that under acid conditions, the 419 negative charge on DCD-1(L) becomes neutral, which facilitates membrane partitioning and Zn2+ 420 dependent membrane channel formation via either a barrel-stave pore or a toroidal pore type 421 mechanism [54]. Another example of AAMPs with pH-dependent activity are kappacin A and 422 kappacin B, which are classed as food peptides and appear to be cleaved from κ -casein in bovine milk 423 by digestion with chymosinin in the human stomach [194]. These peptides exhibit potent activity 424 against a range of Gram-positive and Gram-negative bacteria, including Streptococcus mutans, 425 Porphyromonas gingivalis and Actinomyces lundii [55,56], which make a major contribution to 426 supragingival dental plaques [195,196]. Characterisation studies showed that the antibacterial 427 mechanisms used by kappacins involved the pH-dependent lysis of microbial membranes, which 428 was enhanced by acid conditions [56]. The active region of kappacins included a phosphorylated 429 serine residue that was essential for antibacterial activity [56,197] and interestingly, these peptides 430 showed significantly different pH optima for this activity that resulted from a single residue 431 difference in the sequence of their active regions [55,197]. Kappacin A showed the highest 432 antibacterial activity of the two peptides and possessed an aspartic acid residue in its active region, 433 which was replaced by an alanine residue in the corresponding location of kappacin B [55,197,198]. 434 The functional significance of this difference in sequences is not known although it has been proposed 435 that the additional negative charge possessed by kappacin A may enhance its ability to bind metal 436 ions [9]. It had been demonstrated that the presence of Ca^{2+} and Zn^{2+} ions enhanced the antibacterial 437 activity of kappacins and it was suggested that these ions may form a cationic salt bridge between 438 kappacins and anionic components of the bacterial membrane, thereby facilitating membrane 439 binding and antibacterial action [197]. It has been proposed that the membrane interactive 440 conformation of these peptides may be a proline-kinked amphiphilic α -helix but conformational changes observed in the peptide in the presence of membrane mimic could not be clearly assigned toany particular secondary structures and the structure of kappacins remains unclear [9,56,197,198].

443

444 2.4. Marine invertebrates

445 Myticin C (myt C) exists as a number of isoforms in the bivalve mollusc, *Mytilus galloprovincialis* 446 [199-201] and has been shown to possess activity against fish viruses, including Hemorrhagic 447 Septicaemia virus and Infectious Pancreatic Necrosis virus [202]. Several studies were conducted on 448 the antimicrobial activity of a reduced form of myt C, (myt Cc, [62,63]) based on the proposal that the 449 endogenous reduction of cysteine-stabilized AMPs to produce peptides with higher levels of 450 antimicrobial activity may form part of some innate immune systems [116,117]. It was found that the 451 antiviral activity of myt C, myt Cc and derivatives of both these peptides was enhanced by low pH. 452 These studies also showed that only under acid conditions did these various peptides possess activity 453 against Gram-positive bacteria and Gram-negative bacteria. Structure function studies on E. coli 454 suggested that the antimicrobial action of myt C and its variants was membranolytic and involved 455 low pH mediated increases in their levels of α -helicity and β -hairpin elements within their molecular 456 architecture [62,63]. In addition to their pH dependent antimicrobial activity, both myt C and myt Cc 457 possessed chemotactic activity and appeared to be the first chemokine/cytokine-like molecules 458 identified in bivalves [63,202]. These results added to the increasing evidence that AMPs can serve as 459 cytokines [29,203] and interestingly, it is also becoming clear that these latter peptides are able to 460 exhibit antimicrobial activity [204,205] that is enhanced by low pH [206,207]. Molluscs are also a 461 source of histidine containing AMPs with pH dependent activity, such as the peptide, KPS-1, which 462 was isolated from, Atrina pectinate, and under acid conditions, inhibited the growth of a range of 463 Gram-negative bacteria, including P. Aeruginosa, S. typhimurium and Enterobacter sakazakii [64]. Ci-464 PAP-A22 and Ci-MAM-A24 are representative peptides of the Ci-PAP and Ci-MAM families of 465 AMPs from the solitary tunicate (Sea squirt), Ciona intestinalis [65,66], which appear to be produced 466 in haemocytes and granulocytes of the organism. [208,209]. Both peptides were found to be 467 predominantly α -helical and to possess antimicrobial activity [68,209,210] that appeared to have a 468 pH dependency with optima that varied according to the target microbes [65-68]. In the case of Ci-469 PAP-A22, the activity of the peptide against fungi, Gram-negative and Gram-positive bacteria was 470 enhanced by neutral pH except for B. megaterium which was more efficiently killed by Ci-PAP-A22 471 at acid pH [65]. In contrast, Ci-MAM-A24 showed enhanced activity against B. megaterium, B. subtilis, 472 E. coli and P. aeruginosa at low pH but neutral pH optima for action towards S. aureus, Staphylococcus 473 epidermis, Serratia marsecens and Klebsiella pneumoniae. The peptide was also found to exhibit pH 474 independent antimicrobial activity, killing comparable levels of Yersinia enterocolitica and fungi under 475 low and neutral conditions of pH [66]. The antimicrobial activity of both Ci-PAP-A22 and Ci-MAM-476 A24 appeared to involve a membranolytic mechanism that had characteristics consistent with a 477 'carpet' or 'toroidal pore, type model (Table 1). To help explain the differences in pH dependent 478 antimicrobial activity shown by these peptides it was suggested that histidine mediated variation in 479 their positive charge may facilitate optimal membrane interaction on a species- specific basis [65,66] 480 and of course varying lipid composition will mean differing bacterial systems exhibit different 481 changes to key parameters such as lipid packing at varying pH. Interestingly, Ci-MAM-A24 was 482 found to be more potent than Ci-PAP-A22 and appears to be the first AMP reported to kill an intra-483 amoebic pathogen [67]. It was demonstrate that Ci-MAM-A24 was able to kill Legionella pneumophilia, 484 which is a Gram-negative parasite responsible for Legionnaire's disease [211], whilst the organism 485 was replicating intracellularly in Acanthamoeba castellani [67]. It is well established that A. castellani 486 acts as a vector for this bacterium [212,213], which efficiently replicates in the acidic environment of 487 host amoebal phagosomes [214,215]. Ci-MAM-A24 was also able to kill Mycobacteria, in murine 488 macrophages [210] and these acid fast bacteria are known to replicate in the acidic compartments of 489 these host cells [216]. Given the pH dependent antimicrobial activity of the peptide, it is tempting to

490 speculate that the ability of Ci-MAM-A24 to kill these various bacterial parasites was potentiated by 491 the low pH of their host cell environments. Clavaspirin, clavanins and styelins were isolated from 492 another solitary tunicate, Styela clava and these pH dependent AMPs were found to be rich in both 493 histidine and phenylalanine residues [72,73]. In general, it was found that clavaspirin and clavanins 494 possessed pH dependent antibacterial and antifungal activity [69-71,80] with low pH enhancing the 495 ability of these AMPs to adopt α -helical structure and permeabilize the membranes of these 496 organisms [72,73]. It appeared that the protonation of histidine residues under low pH conditions 497 promoted the ability of these AMPs to target microbial membranes whilst the presence of their 498 glycine and phenylalanine residues provided them with the conformational flexibility and structural 499 hydrophobicity to facilitate bilayer partitioning [74-79]. Styelins, which are rich in phenylalanine 500 residues, were found to show activity against both human bacterial pathogens and marine bacteria, 501 such as Psychrobacter immobilis and Planococcus citreus, [72,217]. The best characterised of these AMPs 502 is styelin D, which possesses α -helical structure and is highly unusual in that it contains twelve post-503 translationally modified residues [81]. For example, the peptide contained multiple 504 bromotryptophan residues, which are found in the AMPs of other marine organisms [218-222] and 505 play an important role in the life of sea sponges and lower marine invertebrates [223]. Styelin D's 506 post-translationally modified residues enhanced the peptide's membranolytic action at low pH but 507 only against Gram-positive bacteria. It was suggested that a role for these extensive modifications 508 may be in preserving activity against certain organisms under the acid conditions found in 509 haemocytes of *S. clava* where the styelins are active [81]. 510

511

512 2.5. Terrestrial invertebrates

513 Hebraein is produced by the tick, Amblyomma hebraeum [224] and showed acid pH optima for its 514 activity against E. coli, S. aureus and the fungus, C. glabrata [82], a major cause of vulvovaginal 515 candidiasis in diabetics [225]. The peptide possessed an α -helical structure except for a short C-516 terminal extension containing multiple histidine residues, which appeared to be required for activity 517 against these organisms [82]. Based on these observations, it was suggested that the acidic pH 518 induced in the physiological environment when a tick blood-feeds would increase the cationicity of 519 hebraein and thereby its membrane interactivity and antimicrobial potency [82]. However, the 520 activity of hebraein against S. aureus appeared to be independent of this histidine cluster and C. 521 albicans was not susceptible to the action of the peptide suggesting that it possessed a variety of 522 antimicrobial mechanisms, which were influenced by the target organism [82]. Interestingly, these 523 latter studies showed hebraein to possess homology and structural similarities to microplusin, which 524 is a Cu²⁺ chelating peptide isolated from another arachnid, the cattle tick, *Riphicephalus microplus*, with 525 broad range antimicrobial activity [226-228]. Studies on the Gram-positive bacterium, Micrococcus 526 luteus, and the fungus, Cryptococcus neoformans, suggested that Cu²⁺ chelation involving histidine 527 residues promotes the antimicrobial activity of microplusin by depriving vital cellular processes of 528 the ion, such as haeme-copper terminal oxidases that contribute to cell respiration [228-230]. 529 Amoebapores are a family of cystein stabilized antimicrobial proteins with α -helical structures that 530 are found in the cytoplasmic granules of the protozoan parasite of primates, Entamoeba histolytica 531 [83,85,231], and interestingly there is evidence to suggest that amoeba-like peptides may have been 532 amongst the first eukaryotic AMPs to emerge [232]. Amoebapore A is the best characterized of this 533 family of proteins and has been shown to exhibit pH dependent activity against Gram-positive 534 organisms, such as M. luteus, and Gram-negative bacteria, including E. coli [83,84,87], which appears 535 to involve pore formation in the membranes of target organisms [84]. Both pore formation and the 536 antibacterial activity of the protein were enhanced by low pH, which appears to derive from an 537 increased ability of amoebapore A to self-associate and form oligomers with some similarities to a 538 barrel-stave pore type mode of action [83-85]. Elucidation of the structure of amoebapore A indicated 539 that a C-terminal histidine residue acted as a molecular switch that triggers the formation of active 540 dimers from inactive monomers, which leads to the construction oligomeric pores in target cell 541 membranes, [86]. In addition to amoebapore A, two isoforms of the protein, amoebapores, B and C, 542 are included in the amoebapore family and all three isoforms differ markedly in their primary 543 structure and spectra of antibacterial activity, synergising their combined efficacy. Amoebapores, B 544 and C are believed to have similar antibacterial mechanisms to amoebapore A and the acidic pH 545 optima of these proteins is consistent with the low pH conditions encountered in the 546 amoebic intracellular vesicles, which form their site of action [83,85,231]. More recently, 547 acanthaporin, which is another protozoan protein with pH dependent antimicrobial activity was 548 described in the Acanthamoeba culbertsoni. At neutral pH, acanthaporin appears to exist as an inactive 549 dimer but low pH triggers the histidine mediated production of monomers and the formation of 550 membrane pores, which promoted the activity of the peptide against a variety of bacteria [88]. 551 Caenopores, also known as saposin-like proteins (SPP), are cystein stabilized helical proteins that are 552 found in the nematode, Caenorhabditis elegans [93,232-235] and are distantly related to amoebapores 553 with which they share structural and functional features [89,92,236,237]. Many of the genes encoding 554 SPP proteins in *C. elegans* are induced in response to microbial challenge [232] and several of their 555 gene products have been reported to exhibit pH dependent antimicrobial activity, including SSP-1 556 [89,90], SPP-3 [91], SPP-5 [92] and SPP-12 [90]. These studies showed that that low pH enhanced the 557 ability of caenopores to kill a wide range of microbes, including Gram-negative bacteria, such as E. 558 coli; Gram-positive bacteria, including Bacillus thuringiensis; yeasts, such as Saccharomyces cerevisiae; 559 and amoebae, including Dictyostelium discoideum. For each of these proteins, antimicrobial activity 560 appeared to be based on an ability to form pores in membranes of target organisms under acid pH 561 conditions and it was suggested that this ability was mediated by the multiple internal 562 histidine residues possessed by caenopores. Due to these residues, the positive charge of these 563 proteins is enhanced under acid conditions, increasing the potential for interaction with anionic 564 components of microbial membranes and possibly mediating pore formation, as described for 565 amoebapores [90-93,236,237]. It was observed that the pH dependent activity of these proteins would 566 appear to reflect the pH conditions at the site of their functional action, such as SPP-1 and SPP-5, 567 which are active in the acidic environment of the *C. elegans* intestine [92,93,236,237].

568

569 3. Potential applications of pH dependent antimicrobial peptides and proteins

570 In response to the growing demand for new antibiotics with novel mechanisms of action, the 571 number of AMPs and antimicrobial proteins entering clinical trials is accelerating [12] and included 572 within these antimicrobial molecules are a number that have been reviewed here (Table 2). Currently, 573 the only pH dependent anionic AMPs that appear to have been commercially developed are 574 kappacins. Based on their activity against oral pathogens [55,56], preparations including these 575 peptides and zinc have been patented [238] and are available as a dental care products [194,198]. It 576 has also been shown that these peptides exhibit increased antimicrobial activity in foods with high 577 calcium contents [194], which, taken with the history of the safe use of κ -casein, led to the proposal 578 that kappacins may be used as a preservative [239]. In the case PD-3-7, epimers of this peptide appear 579 to be the only amyloid forming amphibian anionic AMPs so far reported and have the potential to 580 progress understanding of the role of residue chirality in the formation of disease-related amyloid 581 and aid the design of amyloid-based nanomaterials [126]. The development of functional amyloids 582 as novel nanostructure materials for multiple purposes, such as drug delivery and tissue repair / 583 engineering, is a growing area of technology [240,241] and recently, techniques have been developed 584 to detect epimeric AMPs in the complex skin secretions of frogs and toads [242].

585 The most researched of the cationic AMPs reviewed here for potential medical development is 586 LL-37, which is a prospective broad range antimicrobial agent that is also able to induce wound 587 healing and angiogenesis as well as modulate apoptosis [243]. This potential is though limited in 588 some cases by the pleiotropic effects of the peptide [244]. For example, the peptide shows a variation 589 in its sensitivity to cancer types, promoting proliferation, migration, and tumorigenesis in breast, 590 lung, and prostate cancers through receptor signaling but suppresses proliferation and induces 591 apoptotic and autophagic cell death in gastric cancer, colon cancer, and T-cell leukemia [107]. 592 However, wound treatment is a globally prevalent and economic burden, which makes the 593 pleiotropic ability of LL-37 to exert healing properties and combat multiple microbial pathogens an 594 attractive platform that has been used to develop potential therapeutic strategies for wound 595 treatment [245]. For example, a clinical phase I/II study conducted by Pergamum on LL-37 led to a 596 patent [246] and showed that topical application of the peptide was safe and enhanced wound 597 healing in patients with chronic venous leg ulcers and diabetic patients suffering from infected 598 wounds [245,247]. Wound infection is a major complication in diabetic patients and in particular, 599 infected foot ulcers is one of the most serious and frequent of these complications, which accounts 600 for over 50% of all lower limb amputations performed on these patients [248]. More recently, several 601 studies have developed biodegradable drug delivery system that facilitated the controlled sustained 602 release of LL-37 and other wound healing agents, such as lactate and serpin A1, from nanoparticles. 603 LL-37 and these agents acted synergistically in the treatment of full thickness excisional wounds, 604 significantly promoting wound closure, reducing bacterial contamination and enhancing anti-605 inflammatory activity. These systems offered several advantages over therapies commonly used to 606 treat chronic wound infections, which are often limited due to factors, such as the lack of controlled 607 delivery and the depth of skin infections [249,250]. A number of LL-37 related peptides have also 608 shown the potential for therapeutic development [243,251], such as OP-145, which was developed by 609 OctoPlus, and when the peptide was included in cream formulations for nasal application, these 610 preparations were found to be efficacious in the eradication of MRSA carriage [252]. The anterior 611 nares are the main reservoir for colonization by S. aureus and the nasal carriage of MRSA is an 612 important risk factor for subsequent infection and transmission of this pathogen, which has led to 613 intensive efforts to identify agents able to efficiently reduce MRSA colonization [253]. The completion 614 of phase I/II clinical trials by OP-145 also showed that the peptide was safe and efficacious as a 615 treatment for chronic otitis media, or chronic bacterial middle-ear infection (Table 2) [254]. This 616 disease afflicts millions of people worldwide and is highly recalcitrant to treatment by conventional 617 antibiotics, which is now known to be primarily due to bacterial biofilms [255]. Another derivative 618 of LL-37, 60.4Ac, has also proven to be beneficial in the treatment of patients with otitis media [245] 619 and more recently the peptide showed the potential for development as a novel local therapy to treat 620 patients with burn wounds infected with multidrug-resistant bacteria, including MRSA [256]. Burn 621 wounds are one of the most common and devastating forms of trauma and the infection of these 622 wound by drug resistant bacterial pathogens is rapidly becoming a serious therapeutic challenge in 623 the care of burn patients [257].

624 Histatins and their derivatives show the potential for a wide range of therapeutic and 625 biotechnical application [141], particularly in the field of dentistry and bio-dental research [258]. For 626 example, the hst-5 derivative, JH8194, is a promising candidate to act as a surface substrate in dental 627 implants to prevent peri-implantitis and peri-implant mucositis whilst decreasing infections 628 [259,260]. A major focus in the medical development of histatins has been in the preparation of 629 formulations to treat oral diseases and infections [141]. For example, highly effective hydrogel 630 delivery systems for the topical and oral application of hst-5 have been developed for the treatment 631 of oral candidiasis [261], which is the most common opportunistic fungal infection in 632 immunocompromised populations [262]. High potential for the topical treatment of this fungal 633 condition was also demonstrated when derivatives of hst-5 were conjugated to spermidine and tested 634 on immunocompromised murine models [263]. Compared to hst-5, these conjugates exhibited a 635 higher clinical half-life, enhanced uptake into *Candida* cells, and greater candidacidal efficacies, and 636 were proposed to be viable alternatives to azole antifungals [263], which are commonly used to treat 637 oral candidiasis [262]. A compound derived from hst-5 and hs1-3, P-113 (PAC-113), developed by 638 Pacgen, was evaluated in Phase 1/ II clinical studies for the treatment of both oral candidiasis and

639 gingivitis, and was found to be safe and effective in the treatment of both conditions (Table 2)[12]. 640 Gingivitis is the most common form of periodontal disease, affecting up to 15% of the adult 641 populations worldwide and primarily due to Porphyromonas gingivalis. Untreated the condition can 642 lead to periodontitis, the chronic destruction of connective tissues, and ultimately result in loss of 643 teeth [264]. P-113 has been patented [265] and most recently, it has been shown that the candicidal 644 efficacy of the peptide was greatly enhanced when it was modified by coupling to other AMPs and 645 their derivatives [144] Another histatin, hist-1, was conjugated to a silver metallopharmaceutical and 646 the conjugate was found to have wound healing properties coupled to potent activity against 647 bacteria, which included MRSA, indicating the potential for development of novel multifunctional 648 therapeutics [266]. Clavanins are attractive candidates for development as drugs against bacteria 649 associated with sepsis, which is rapidly becoming a problematic nosocomial infection [267], and 650 recently developed nanoparticle formulations of these peptides, showed high promise as a drugs 651 against polymicrobial sepsis with morphological characteristics suitable for administration via 652 injection [80]. Derivatives of clavanins have also been developed to combat biofilms formed by, S. 653 *mutans*, which is a major contributor to dental plaque and one of the major etiological factors involved 654 in causing caries [268]. Dental caries is one of the most prevalent, preventable infectious diseases 655 affecting humans and is recognized as the primary cause of oral pain and tooth loss [269].

656 The major medical development of the antimicrobial proteins reviewed here appears to be 657 lactoferrin, which as described above is an iron binding protein but, like LL-37, is pleiotropic and also 658 displays broad range antimicrobial activity using a number of mechanisms, which includes the 659 release of derivative AMPs via hydrolysis by proteases [162,270,271]. Lactoferrin and its related 660 peptides shows the potential for a number of clinical uses, ranging from wound healing and the 661 detection of bacteria to the treatment of microbial infections both alone and in combination with other 662 clinically relevant agents [272]. A full description of these medical uses is beyond the scope of this 663 review but lactoferrin and its derivatives have featured in multiple clinical trials [160,272,273] and 664 have numerous entries in a recently constructed database of bioactive peptides derived from milk 665 proteins [274]. As major examples, lactoferrin and its derivatives have been extensively investigated 666 as potential drugs for the treatment of common viral infections including the common cold, influenza, 667 viral gastroenteritis and herpes [275] whilst the inhibitory effects of these proteins and peptides 668 against the proliferation of multiple cancers, has suggested a potential role in cancer prevention [276]. 669 It is well established that many AMPs and antimicrobial proteins have anticancer activity that 670 generally appears to involve mechanisms of membranolysis that are similar to those used by these 671 molecules in their action against microbes [277,278], which in some cases shows pH dependence [98], 672 as recently described [279,280]. Advanced clinical trials have shown that the administration of 673 lactoferrin has no significant side effects and that the protein has efficacy in treating iron deficiency 674 anemia in pregnant women [281], sepsis in premature neonates, which is a common and severe 675 complication in new-born infants [282] and infections due to Helicobacter pylori, which is causally 676 associated with gastritis and peptic ulcer diseases [283]. A major example of the medical potential of 677 lactoferrin is the development of ALX-109 by Alaxia, which is a combination of the protein and 678 hypothiocyanite for the treatment of CF [160]. This drug combination has been granted orphan drug 679 status by American and European licensing agencies and has been shown to enhance the ability of 680 conventional antibiotics to eliminate biofilms of *P. aeruginosa* growing on CF airway epithelial cells 681 [284]. Derivatives of lactoferrin, have also shown the potential for therapeutic development such as 682 hLF(1-11), which was developed by AM Pharma, and in clinical trials the peptide was safely injected 683 into neutropenic stem cell transplantation patients [160]. Neutropenia is defined as a reduction in the 684 absolute number of neutrophils in the blood circulation, predisposing individuals to severe or fatal 685 infections [285], and currently, hLF(1-11), awaits development for the prevention of bacteremia and 686 fungal infections in immunocompromised individuals (Table 2)[273]. Lactoferricin B is cleaved from 687 the N-terminal region of bovine lactoferrin under acid pH conditions and has an extremely wide 688 spectrum of antimicrobial activity against bacterial, fungal and parasite species as well as showing 689 anti-catabolic and anti-inflammatory effects [162,270,271]. Based on these abilities, this peptide has

690 featured in numerous preclinical trials and shows the potential for a variety of therapeutic purposes,

691 including the treatment of ocular infections, osteo-articular gastro-Intestinal and dematological

diseases, along with applications in veterinary practice and the food industry [272]. The commercialimportance of lactoferrin and its derivatives is perhaps underlined by the fact that the recombinant

693 importance of lactoferrin and its derivatives is perhaps underlined by the fact that the recombinant 694 human protein has been expressed in transgenic cattle to provide the large-scale production of

- 695 lactoferrin for pharmaceutical use [286]. The recombinant protein has also been expressed in microbes
- and higher plants in the search for bioreactors with the capacity for large-scale production, which,
- 697 led to lactoferrin expression also being used as a tool for the enhancement of plant resistance to
- 698 pathogens [286].
- 699

700 4. Discussion

701 AMPs and antimicrobial proteins with pH dependent action against microbes appear to receive 702 relatively little attention in the literature but, as this review has shown, these molecules are produced 703 by a diverse spectrum of eukaryotes, including: vertebrates, such as fish, humans, horses, cattle, 704 rabbits, guinea pigs, mice, frogs and toads, as well as invertebrates, such as ticks, parasites, worms 705 and mollusks (Table 3). Around two thirds of the molecules reviewed here are cationic AMPs and 706 antimicrobial proteins with most of those that remain possessing net negative charges [287]. It is 707 generally recognized that the incidence of anionic antimicrobial molecules is low and that, in general, 708 their occurrence appears to be a strategy to synergize the antimicrobial activity of their cationic 709 counterparts [9,288]. For example, the proteolytic processing of the sweat borne peptide, dermcidin, 710 to yield DCD-1(L), described above, also produces a number of other anionic AMPs, such as SSL-46 711 (net charge –2) and LEK-45 (net charge -2) [289]. These sweat-derived anionic AMPs are continually 712 secreted and are believed to synergize the activity of cationic AMPs in the constitutive innate defense 713 of human skin by modulating surface colonization by microbes rather than responding to injury and 714 inflammation as observed for inducible peptides, such as LL- 37 [186].

715 The pH dependence of the antimicrobial molecules reviewed here was found to vary with pH 716 with some, such as E2EM-lin, exhibiting high pH optima (Table 4) whilst others, such as Ci-PAP-A22 717 and Ci-MAM-A24, exhibited optima at either neutral or acid pH depending on the target organisms 718 [65-68]. Again depending on the target microbes, several antimicrobial molecules, including the latter 719 peptides and psiorasin, showed the ability to employ both pH dependent and pH independent 720 activity [38-40,65-68]. However, most of the AMPs and antimicrobial proteins reviewed here 721 exhibited low pH optima, which is consistent with the acidic pH found at their sites of action, 722 particularly the skin [131,290]. Consistent with these observations, the major structure / function 723 relationships that promote the pH dependent activity of the antimicrobial molecules reviewed here 724 are those involving amino acid residues that become protonated under acid conditions, including 725 histidine, aspartic acid and glutamic acid residues. Under these pH conditions, the protonation of 726 these residues will have the overall effect of increasing the cationicity or decreasing the anionicity of 727 the parent molecule thereby, enhancing its ability to target and interact with negatively charged 728 components of microbial membranes. Typical examples include hebraein [224] and clavanins [74-79], 729 and in the case of Ci-PAP-A22 and Ci-MAM-A24, it appears that the histidine mediated variation in 730 the cationicity of these peptides facilitates optimal interaction with target microbial membranes on a 731 species to specific basis [65,66]. However, given the high incidence of histidine residues in the 732 antimicrobial molecules reviewed here, it is worthy of note that the possession of these residues is 733 not necessarily sufficient for a pH dependent mode of antimicrobial action. This point is well 734 illustrated by Pc-pis, from the yellow croaker, Pseudosciaena crocea, which includes a number of 735 histidine residues in its primary structure and displays pH independent antimicrobial activity. 736 However, the addition of a histidine residue to its sequence generated a peptide with antimicrobial 737 activity optimal at low pH and a wider spectrum of antimicrobial activity [291].

738 A second major structure / function relationship for histidine, aspartic acid and glutamic acid 739 residues in the antimicrobial action of pH dependent AMPs and proteins reviewed here is to facilitate 740 the binding of metal ions. For example, the binding of Ca2+ by MSP at low pH potentiates the activity 741 of the peptide by alleviating inhibitory mechanisms that are mediated by the ion [41] and metal ion 742 binding by histidine residues appears able to promote microbial death through depletion of these 743 ions for a number of antimicrobial molecules, such as histatins [141,143,155]. In contrast, the binding 744 of metal ions appears to potentiates the activity of some antimicrobial molecules reviewed here by 745 promoting their capacity to form peptide-membrane or peptide-peptide salt bridges and thereby 746 disrupt microbial membranes, as proposed for kappacins [197] and DCD-1(L) respectively [54]. 747 However, the most common structure / function relationships for histidine, aspartic acid and 748 glutamic acid residues in the antimicrobial action of the molecules reviewed here is to directly 749 promote the disruption of target microbial membranes. For example, in the case of several 750 antimicrobial proteins, the protonation of histidine residues appears to be a molecular switch that 751 initiates oligomerisation and the formation of discrete channels or pores by the protein, as in the case 752 of acanthaporin [88]. In some cases though, histidine, aspartic acid and glutamic acid residues appear 753 to play multiple roles in promoting the activity of their parent antimicrobial molecules. For example, 754 the N-terminal regions of gad-1 and gad-2 include a number of sequential histidine pairs that appear 755 to be important to their ability for lipid targeting and interaction, channel formation and thereby the 756 disruption of microbial membranes at low pH [33,34,94,99,100].

- 757 A further major structure / function relationship involved in the mechanisms of the antimicrobial 758 molecules reviewed here is pH related conformational change in α -helical architecture, which is by 759 far the most common secondary structural element identified in these AMPs and antimicrobial 760 proteins. Indeed, it well established that histidine, glutamic acid and aspartic acid residues have a 761 strong potential for α -helical formation that is enhanced by low pH [98,292]. The pore forming 762 antimicrobial proteins reviewed here are strongly α -helical (Figure 2) and it is known that changes to 763 the levels of α -helical architecture possessed by these proteins are enhanced by low pH, which 764 promotes their pore forming mechanisms and are key to their ability to kill microbes [39,86,88,93]. A 765 full description of these conformational changes is beyond the scope of this review but as an example, 766 the protonation of C-terminal histidine residues by low pH promotes conformational changes that 767 lead to the construction of hexameric membrane pores via the formation of active dimers from 768 inactive monomers in the case of amoebapores [83,85,231] caenopores [89-93] and psoriasin [38-40]. 769 The pore forming mechanism of acanthaporin, shows similarities to those of these latter proteins and 770 also results in the formation of hexameric membrane pores. However, in the case of acanthaporin, 771 the low pH mediated protonation of C-terminal histidine residues promotes conformational changes 772 that induce pore formation via the formation of active dimers from inactive monomers [88]. Strictly, 773 based on is size, lactoferrin is an antimicrobial protein, but it is often classified with AMPs due to its 774 ubiquity in body fluids and its ability to kill bacteria using membrane interactive mechanisms with 775 similarities to those of these latter peptides [29]. However, lactoferrin was first characterized as an 776 iron binding protein and sequestration of the metal was initially believed to form the basis of its 777 antibacterial mechanism although the protein is now known to use multiple iron-independent 778 mechanisms in its activity against microbes [162,293].
- 779 In relation to the AMPs reviewed here low pH generally increased their levels of α -helical 780 secondary structure and thereby enhanced their capacity for membrane interaction and antimicrobial 781 activity. However, alkaline conditions promoted maximal levels of α -helical structure in E2EM-lin, 782 which appeared to promote monomer association, pore formation and membrane interaction at the 783 peptide's high pH optimum (Table 4, Figure 1). In contrast, to these latter AMPs, gad-1 and gad-2 784 were found to possess minimal levels of α -helical structure under the low pH conditions that were 785 optimal for their membrane interactions and antimicrobial activity [33]. These observations would 786 seem to clearly indicate that pH dependent structural plasticity is an important factor in the 787 antimicrobial mechanisms of many of the AMPs reviewed here. This form of structural plasticity 788 would appear to be key to facilitating the appropriate balance between the amphiphilicity and

hydrophobicity of these peptides that is required for their membranolytic action at optimal pH, as proposed for gad-1 and gad-2 [33]. Reinforcing this proposal, other amino acid residues have been reported to contribute to the structural plasticity of the α -helical AMPs reviewed here including glycine, phenylalanine and post-translationally modified residues. These residues appear to enhance the conformational flexibility and structural hydrophobicity of tunicate clavaspirin, clavanins and styelins for bilayer partitioning and antimicrobial action at their low pH optimum [72,73].

795 The antimicrobial mechanisms of several AMPs reviewed here appear to be described by models 796 of membrane interaction, including variants of the carpet, toroidal pore and barrel-stave pore 797 mechanisms. These models of membrane interaction differ fundamentally to the pore forming 798 mechanisms of the antimicrobial proteins described above and were primarily proposed to describe 799 the membrane spanning abilities of AMPs, which are generally up to 50 residues in length [23]. A 800 number of novel antimicrobial mechanisms for AMPs have also been revealed by this review, such 801 as that described for human lactoferrin, which at sub lethal levels appears to kill microbes via the pH 802 dependent dissipation of microbial PMF [51]. The microbial PMF is an emerging potential target for 803 the development of novel AMPs and antimicrobial proteins based on the fact that the temporary 804 membrane perturbations caused by their action can have a large negative impact on bacterial 805 metabolism, affecting a diverse array of cellular processes that depend upon the PMF [294-299]. This 806 review has also described novel examples of pH dependent AMPs produced by the reduction of 807 cysteine stabilized parent AMPs including myt Cc [63,202] and E2EM-lin (Table 4, Figure 1, [114,115]) 808 and it has been recently shown that the free cysteines of reduced AMPs play an important role in 809 their antimicrobial activity [116,117]. Moreover, it was speculated above that the antimicrobial 810 activity of E2EM-lin may involve pore formation via self-association and interestingly, recent work 811 has suggested that free cysteine residues may play a role in the antimicrobial activity of AMPs by 812 facilitating the oligomerisation of these peptides [300]. Taken together, these reports show that AMPs 813 with pH dependent antimicrobial activity contribute to the accumulating evidence that the 814 endogenous reduction of cysteine-stabilized AMPs is a strategy used by hosts to generate novel 815 peptides that enhance the efficacy of their antimicrobial capacity [116,117].

816 A number of the AMPs and antimicrobial proteins reviewed here, along with their derivatives, 817 have been developed for multiple medical purposes, which in some cases has led to patents and the 818 successful completion of clinical trials, and include kappacins, LL-37, histatins, lactoferrin and 819 clavanins. Major examples of the application of these AMPs and proteins include the treatment of 820 multiple cancers along with viral infections, such as the common cold; bacterial infections, including 821 those associated with implants, otitis media, neutropenia and CF; and fungal infections, particularly, 822 those detrimental to oral health. These AMPs and proteins also show the potential to induce wound 823 healing, such as for diabetic patients and burn victims, and interestingly, a recent report has indicated 824 that wound healing is accelerated by an acidic environment, which promotes a range of beneficial 825 effects including increases in antimicrobial activity and the enhancement of epithelization and 826 angiogenesis [301]. In general, the therapeutic administration of the AMPs and proteins involve 827 topical application, such as the use of mouth washes, cream formulations and hydrogel delivery 828 systems. These observations raise an interesting point in that most clinical trials to date involve the 829 treatment of skin infections or the prevention of surface colonization by microbes, particularly sessile 830 forms of these organisms, which potentially, can indicate a wide variation in local pH conditions. A 831 comprehensive understanding of the effect of pH on the antimicrobial activity of the molecule under 832 development would therefore seem necessary. Nonetheless, this is not generally the case and data 833 cited in the literature in relation to the antimicrobial activity of AMPs and proteins is usually that 834 determined under neutral pH conditions [133]. These observations clearly suggest that when 835 characterizing the antimicrobial action of AMPs, the optimal pH for their action against individual 836 microbes should be determined. This point is well illustrated by recent studies, which investigated 837 the antimicrobial action of a range of synthetic AMPs and found that high pH inhibited the action of 838 these peptides against fungi and Gram-negative bacteria but the opposite pH trend was observed 839 for Gram-positive bacteria [302].

840 5. Conclusions

841 This review has shown that AMPs and proteins with pH dependent antimicrobial activity are 842 increasingly being reported and that progress has been made in understanding the structure / 843 function relationships and mechanisms underpinning this activity. This review has also shown that 844 there has been considerable therapeutic development of pH dependent antimicrobial molecules to 845 treat a variety of infections and other conditions. However, one of the biggest therapeutic and 846 biotechnical developments of these antimicrobial molecules has been to provide guidance to the 847 design of novel compounds with pH dependent activity against: bacteria [303,304], fungi [50,305,306] 848 and cancer cells [307,308] as well as applications involving drug [309,310] and gene delivery [311,312]. 849 As a specific example, most AMPs designed to target the low pH of tumor tissue are cationic and 850 cytotoxicity to healthy tissue at physiological pH has often been an issue for these peptides 851 [98,279,280]. To address this issue, a peptide based on magainin 2 from X. laevis was designed to 852 possess a negative charge at neutral pH that switched to a strong positive charge at low pH for cancer 853 targeting. Designated, HE, this novel peptide, killed human renal adenocarcinoma at low pH via 854 membranolytic mechanisms and was nontoxic towards healthy human cells across low and neutral 855 pH conditions, making it a promising lead compound for cancer therapy [313]. As a further example, 856 chronic infections due to *P. aeruginosa* are responsible for the majority of the morbidity and mortality 857 in patients with CF and the persistence of these infections is largely due to the organism adopting a 858 biofilm mode of growth, thereby acquiring high resistance to most antibiotics [314,315]. In response, 859 the peptide, WLBU2, was designed and found able to prevent biofilm formation by *P. aeruginosa* 860 under the low pH and high salt conditions characteristic of the CF airway without negative effects 861 on human airway epithelial cells. WLBU2 was also found able to synergize the action of commonly 862 used antibiotics, such as tobramycin and meropenem, making the peptide an attractive proposition 863 to help address the critical need for novel therapeutics able to suppress chronic CF lung infections 864 [316]. Using another approach, it has also been proposed that increasing the airway pH in CF 865 individuals by activating CFTR independent HCO3⁻ transport pathways or by inhibiting proton 866 pumps could help prevent or reduce bacterial and viral infections associated with the disease 867 [165,180,181]. Nonetheless, this review has shown that many pH dependent AMPs and antimicrobial 868 proteins have yet to be fully characterized and it is proposed that these antimicrobial molecules merit 869 far more research attention than they currently receive. Indeed, pH dependent AMPs and 870 antimicrobial proteins appear to represent an untapped source of novel biologically active agents that 871 is awaiting full exploitation and could aid fulfillment of the urgent need for alternatives to 872 conventional antibiotics, helping to avert a return to the pre-antibiotic era.

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