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Antimicrobial peptides with pH dependent activity and alkaline optima: their origins, mechanisms of action and potential applications

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Abstract: A number of disorders and diseases are associated with conditions of high pH and many conventional antibiotics lose their efficacy under these pH conditions, generating a need for novel antimicrobials, and a potential solution to fulfil this need is antimicrobial peptides (AMPs) with high pH optima. This review shows that a variety of anionic and cationic AMPs with this pH dependency are produced by creatures across the eukaryotic kingdom, including humans, rabbits, cattle, sheep, fish and frogs. These AMPs exhibit activity against viruses, bacteria and fungi that involves membrane interactions and appear to be facilitated by a variety of mechanisms that generally promote passage across membranes to attack intracellular targets, such as DNA or protein synthesis, and / or membrane lysis. Some of these mechanisms are unknown but those elucidated include the use of bacterial pores and transporters, the self-promoted uptake pathway and established models of membrane interaction, such as the carpet mechanism, toroidal pore formation, the adoption of tilted peptide and the SHM model. A variety of potential roles have been proposed for these AMPs, including use as antivirals, antibacterials, antifungals, adjuvants to antimicrobial therapy, biomarkers of disease and probes for pathogenic microbes. In this review, these properties are described and discussed, with an emphasis on the antimicrobial mechanisms used by these AMPs and the pH dependency of these mechanisms.

Keywords: Antimicrobial peptides, pH dependent, alkaline optimum, antibacterial, antiviral, antifungal, membranolysis, pore formation.

1. INTRODUCTION

It is well established that pH plays an important physiological role in humans that is tightly regulated by acid-base homeostasis; however, unregulated changes in pH can impact on human health *via* multiple routes [1]. In particular, a number of disorders and diseases are associated with conditions of high pH [2-4]; for example, bacterial prostatitis, which is a common urological disorder in men [5, 6]. In this disorder, which is commonly caused by *Escherichia coli* and other Gram-negative bacteria of the Enterobacteriaceae family, the pH of the prostatic fluid becomes markedly alkaline [7], and it is believed that this is a major reason for the low efficacy of some antibiotics in treating the condition [6, 8]. High pH is also associated with secretory diarrhoea caused by enterotoxigenic *E. coli* (ETEC) [9, 10], which is a leading cause of morbidity and mortality in children under 5 years old [11, 12]. In this condition, alkaline pH is used as a signal by ETEC to guide colonization of its infection niche, which is close to the epithelium in the small intestine, and to maximize secretion of the toxins responsible for secretory diarrhoea [9, 10]. Alkaline pH is a feature of chronic wounds, which do not heal easily, in part, due to the fact that these pH conditions promote colonization of the wound by microbial biofilms [13, 14]. A number of pathological skin conditions are also associated with alkaline pH, including psoriasis [15], acne [16] and, notably, atopic dermatitis (atopic eczema), which is a chronic, pruritic inflammatory skin disease [17]. The elevated

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pH accompanying these conditions appeared to result from the influence of both endogenous factors, such as a dysregulated skin buffering capacity, and exogenous factors, including age, body part, and skin type [18-20]. These factors collaborate to promote inflammation and microbial colonization [21], primarily by *S. aureus*, which is exacerbated by the decreased production of endogenous antimicrobials and leads to dysbiosis of the skin microbiome [22-24].

A number of antibiotics, including aminoglycosides and macrolides, have optimal efficacy under alkaline pH conditions; however, many others, such as tetracyclines and β-lactams, show their highest activity under acidic pH conditions [25-27]. The clear need for antibiotics with alkaline pH optima has been further exacerbated by the general paucity of novel antibiotics under development, which are generally modifications of existing classes of these molecules [28-30]. In response, there is an ongoing search for new antibiotics with novel mechanisms of action and a major focus of this search is antimicrobial peptides (AMPs), which are endogenous antibiotics that are produced by most living organisms [30, 31]. It is well established that most AMPs possess cationic, amphiphilic structures that promote their ability to target and interact with anionic components of microbial membranes, which leads to membranolysis [32, 33] and / or translocation across bilayer to attack intracellular targets [34, 35]. Similarly to conventional antibiotics [25-27], the activity of many AMPs shows sensitivity to pH [36], with most of these peptides possessing acid pH optima [37] and a smaller number exhibiting alkaline optima (Table1). However, in contrast, AMPs are much less likely to induce resistance in target microbial cells, primarily due to the non-specific nature and multiple targeting capacity of their membranolytic mechanisms, as compared to the single site of action generally used by conventional antibiotics [38-40]. This decreased likelihood of bacterial resistance gives AMPs a major advantage as potential therapeutic and biotechnical agents, and the capacity to serve in these contexts has recently been reviewed for these peptides that exhibit acid optima in their antimicrobial action [37]. However, no corresponding review of AMPs with alkaline optima for their antimicrobial activities appears to be in the literature, and in response, the current study presents an overview of these peptides with a focus on their mechanisms of action and potential for application.

1.1. AMPs from mammals

It is a matter of debate as to when AMPs were first reported [41] but clear contenders would appear to be the pH dependent, mammalian peptides described by Hirsch and colleagues in the middle of the 1950s [42-45]. These studies are considered as a milestone in the history of AMPs, predating by almost three decades the seminal work of the early 1980s, which is generally taken as the beginning of front line research into these peptides [41, 46, 47]. The first of the AMPs described by Hirsch and colleagues was a strongly cationic peptide found to be present in the thymus of calves and sheep, as well as a variety of other calf organs [42, 43]. Antibacterial assay showed that this peptide exhibited non-membranolytic activity against *Mycobacterium tuberculosis*, the etiological agent of human tuberculosis [48], that was enhanced by alkaline pH, which would appear to be the first report of AMPs with this form of pH dependency [37]. A limited characterization of the calf thymus peptide showed that it contained a high level of arginine and lysine residues, which were predominantly responsible for its strong positive charge and its ability to target bacteria [42, 43]. These compositional analyses also showed that the peptide contained no cystine residues [42, 43], precluding it from the defensin family of AMPs, which are β-sheet molecules with multiple disulphide bridges that are produced by cattle, sheep and a variety of other creatures [41, 49, 50]. As a matter of historical interest, the second of the peptides described by Hirsch and colleagues, named phagocytin, was present in humans, rabbits, horses and guinea pigs and would appear to be the first example of AMPs with antibacterial activity that was enhanced by acid pH [44, 45]. Phagocytin was found to be active against a variety of Gram-negative and Gram-positive bacteria [44, 45], whilst further investigations established that the peptide was confined to the cytoplasmic granules of neutrophils [51-53]. These studies were one of the first indicators that neutrophils are key effector molecules of the innate immune system [54, 55] and are regarded as a landmark in leucocyte biology [56]. However, surprisingly, no further major research on phagocytin appears to have been conducted [57], with a recent report observing that it is not even known whether this antibacterial agent was rediscovered later and named otherwise [58].

In the mid-1980s, human neutrophil defensin 1 (HNP-1) was obtained from human granulocytes [59] and higher pH was shown to enhance its ability to inactivate herpes simplex virus type 1 (HSV-1) [60], which is an enveloped DNA virus that primarily causes oral herpes [61]. HNP-1 is cationic and the inactivation of HSV-1 by the peptide appeared to involve binding to anionic components of the lipid bilayer possessed by the viral envelope, and the ability to penetrate this bilayer [60]. Around this time, MCP-1 and MCP-2, which showed structural homology to HNP-1 [60], were identified in the alveolar macrophages of rabbits [62] and these peptides also showed an ability to inactivate HSV-1 that was enhanced by alkaline pH [63]. Inactivation of this virus by these cationic peptides appeared to involve direct interaction with anionic components of the lipid bilayer of the viral envelope, leading to membrane disruption that rendered the virus non-infectious [64]. Soon after this work, six cationic peptides were isolated from rabbit neutrophils that possessed activity against a spectrum of Gram-positive and Gram-negative bacteria and included NP-1 and NP-2 [65]. These AMPs were shown to be identical to MCP-1 and MCP-2 [57], possessing the characteristic structures of α-defensins: triple-stranded β-sheet structure that are stabilized by three disulphide bonds [57, 66]. In the case of NP-1, the peptide showed activity against *P. aeruginosa* that was enhanced by high pH [67], and more recent studies have shown that the action of the latter peptide and NP-2 against Gram-negative bacteria appears to involve membrane binding *via* interactions with anionic lipid that leads to permeabilization through the formation of transient lesions [68, 69]. These observations were suggestive of the toroidal pore model, in which AMPs bind the bacterial membrane surface in a parallel orientation *via* electrostatic interactions, which leads to membrane thinning and at a critical concentration, these peptides realign perpendicular to the bilayer, causing the membrane surface to cavitate inwards and ultimately form a pore, which can be transient in nature [70-72]. Interestingly, these peptides were ineffective against *P. aeruginosa* under

acid conditions but were able to permeabilise its outer membrane (OM), which led to the proposal that this ability may serve to synergise the antibacterial action of other defence molecules under the acid conditions associated with phagocytosis [73, 74]. NP-1 and HNP-1 have also been shown to have membranolytic activity against *M. tuberculosis* that was enhanced by alkaline conditions [75] and it has been proposed that these AMPs, as well as other defensins, have the potential for use in the treatment of infections due to *M. tuberculosis* [76]. Indeed, it is becoming increasingly clear that in addition to causing tuberculosis, *M. tuberculosis* is associated with a variety of other diseases, ranging from pulmonary complications to metabolic syndromes [48].

At the time of these studies on HNP-1, NP1 and NP2, mechanisms underpinning the antimicrobial action of these peptides was poorly understood [57, 60, 63, 65, 67, 75]; however, it is now known that the ability of α-defensins to interact with membranes is generally mediated by the tertiary amphiphilicity of their triple-stranded β-sheet structures [57, 66]. In this form of amphiphilicity, residues that are distal in the primary structure of AMPs such as α-defensins are brought together in their tertiary structure to form polar and apolar sites on the molecular surface [72]. These polar sites include cationic and hydrophilic residues that facilitate the targeting and binding of these AMPs to anionic components of microbial membranes, whilst these apolar sites are formed by hydrophobic residues that associate with the acyl chain region of these membranes [72]. In combination, these interactions are able to promote not only the toroidal pore model described for HNP-1, NP1 and NP2 above [70-72], but also other membranolytic mechanisms, such as the barrel-stave, toroidal pore, carpet, and Shai–Matsuzaki–Huang (SHM) models, each of which are described below [33, 72, 77].

In addition to rabbits and humans, α-defensins are now known to be produced by a variety of other mammals, including primates and rodents [57, 58, 74, 78, 79], where they serve not only antimicrobial roles but also contribute to other innate immune functions, such a immunomodulation and stimulating wound repair [80-83]. Based on these roles, α-defensins have the potential for a number of applications, including as antivirals [84], as antibacterials [85],as biomarkers of prosthetic joint infections [86], and as biomarkers and therapeutics for cancer [82, 87]. Interestingly, there is increasing evidence that, in certain biological settings, α-defensins can promote tumorigenesis, as well as viral and bacterial infections, and an understanding of these processes should lead to new therapeutic interventions to treat these conditions [88].

A number of AMPs belonging to the cathelicidin family have been identified in sheep, including Oabac5m Oabac7.5 and SMAP-29 [89-91], which were originally predicted from ovine myeloid cDNA [92-94]; however, it is now known that the latter peptide is post translationally modified to yield its native, amidated form: SMAP-28 [95]. This has led to some confusion in the literature, with the native form of the peptide often being referred to as SMAP-29 rather than SMAP-28; therefore, for clarity, in these cases we refer to SMAP-29 (SMAP-28) in the following discussion [70]. Alkaline pH has been shown to enhance the activity of Oabac5mini and Oabac7.5mini, which are truncated forms of Oabac5m and Oabac7.5 respectively, along with that of SMAP-29 (SMAP-28), against *Escherichia coli* 0157:H7 [96], which causes diarrheal illnesses in humans [97, 98]. In the case of SMAP-29 (SMAP-28), the peptide adopted α-helical structure and killed *E. coli* using mechanisms that appeared to involve high affinity binding to lipopolysaccharide (LPS) [99] and translocation of the OM using the self-promoted pathway [91, 99, 100], which is consistent with more recent studies [101]. LPS is the major component of the OM [102] and using this pathway, the binding of AMPs to this lipid displaces divalent ions that help maintain the stability of the membrane, thereby causing transient permeabilization that allows these peptides to cross the membrane, and gain access to the periplasmic space and inner membrane [77, 103]. Upon accessing the *E. coli* inner membrane SMAP-29 (SMAP-28) appeared to induce disruption of this membrane that promoted a membranolytic mode of bacterial killing [91, 99, 100], which was consistent with other studies on the action of the peptide against both the latter organism and other Gram-negative bacteria [70, 71, 101]. It is generally accepted that the antibacterial and membranolytic activity of SMAP-29 (SMAP-28) is based on its ability to underdo conformational change and adopt α-helical structure [70, 71], contrasting to α-defensins [57, 66]. As with most α-helix forming AMPs, the adoption of this structure by SMAP-29 (SMAP-28) requires the environment of a membrane interface and produces α-helical structure with secondary amphiphilicity that is characterized by a spatial segregation of hydrophobic and hydrophilic residues about the α-helical long axis [70-72]. During membrane interaction, this form of amphiphilicity allows the non-polar α-helical face of AMPs to interact with the membrane lipid core whilst concomitantly permitting its hydrophilic face to engage in electrostatic interactions with the membrane lipid headgroup region. [33, 72, 77]. In combination, these electrostatic and hydrophobic interactions promote the membranolytic and antibacterial action of SMAP-29 (SMAP-28), which has been proposed to involve use of the carpet mechanism [47, 70, 71]. According to this mechanism, AMPs carpet the bacterial membrane surface in a parallel orientation *via* electrostatic interactions up to a threshold concentration when they re-orientate to interact with the membrane core regions, which ultimately leads to membrane fragmentation *via* a detergent like action [70-72].

OaBac5mini and OaBac7.5mini belong to a group of proline-rich cathelicidins known as bactenicins that have been identified in other ruminants, including cows and goats, and similarly to SMAP-29 (SMAP-28), exhibited conformational flexibility, forming polyproline type II helices [89-91, 104]. OaBac5mini and OaBac7.5mini showed no evidence of membranolytic action against *E. coli* but were able to bind LPS and appeared to translocate the OM to access the periplasmic space and inner membrane of the organism [99, 105]. These results were consistent with previous studies [91], and there is evidence to suggest that these peptides translocate the OM of *E. coli* using the self-promoted pathway, described above [91, 99, 105]. In the case of OaBac5mini, the peptide appeared to kill *E. coli* using intracellular sites of action that were proposed to include protein and DNA synthesis [99, 105], which was supported by other studies [34, 106], although mechanisms used by the peptide to cross the inner membrane of the organism were not determined [99, 105]. However, a close bovine homologue of OaBac5mini appeared to kill *E. coli* using non-membranolytic mechanisms that involved internalization *via* SbmA [107-109], an inner membrane transporter [110], and the

blocking of protein synthesis, facilitated by the homologue's extended, polyproline helical structure [107-109]. The use of Sbma and MdtM, which is also an inner membrane transporter [111], facilitates the antibacterial action other proline-rich AMPs, and it has been suggested that OaBac5mini and OaBac7.5mini may use similar uptake mechanisms [34, 104, 112, 113]. Interestingly, Sbma is of unknown physiological function [110], but MdtM is known to be an efflux pump that contributes to the intrinsic resistance of *E. coli* to drugs and antimicrobials by extruding these compounds from the cytoplasm [111, 114]. This co-option of transporters involved in microbial defence mechanisms to facilitate the activity of AMPs against the host microbes has been reported in a number of other cases [34, 35, 104] and would seem to represent an adaptation strategy in the ongoing co-evolution of microbial pathogens and their hosts [115].

A number of potential uses for SMAP-29 (SMAP-28) have been proposed; for example, serving as a preservative for meat products [96]; a variety of microorganism with tolerance to high pH are known to act as food spoilage organisms [116]. Based on the high affinity of SMAP-29 (SMAP-28) for LPS and its activity against Gram-negative bacteria [91, 99, 100], a number of investigations, including animal studies [71, 117-119], have indicated that the peptide is able to confer protection from bacterial infections and sepsis, as well as reducing proinflammatory responses induced by endotoxins [70, 71]. In another therapeutic context, a derivative of SMAP-29 (SMAP-28) was coupled to a fluorescent dye and investigated for use as an agent to detect *E. coli* O157:H7 [120]. This methodology showed a sensitivity comparable to that of labelled antibodies [120], which is a commonly used method to detect Gram-negative pathogens such as *E. coli* O157:H7 but suffers from limitations [121]. More recently, segments of SMAP-28 / SMAP-29 were immobilized onto a functionalized gold surface and the resulting chips showed high accuracy in discriminating between LPS samples from bacterial species, including strains of the same species [122]. For example, this chip correctly differentiated *E. coli* O157:H7 and other pathogenic strains of the organism from non-pathogenic strains, such as *E. coli* K12 [122], which is clearly of significance to the clinical diagnosis and reduction in illnesses due to *E. coli* [97, 98].

In the case of OaBac5mini and OaBac7.5mini, a number of potential uses have been proposed for these peptides, including the protection of meat products [96] and as agents to promote the action of other AMPs and antibiotics against *E. coli* O157:H7 [96, 123]. Similar synergistic results were reported for the action of OaBac5mini when directed against MRSA [123] and previous investigations have shown that the peptide has activity against this organism [99] that appears to involve intracellular sites of action [124]. These results were consistent with those of other studies, which showed that membrane perturbation by AMPs can enhance the uptake of other antimicrobials, and it has been proposed that such combination therapy could reduce the development of microbial drug resistance in medical practice [125-127]. In addition, OaBac5mini has been shown to suppress the production of pro-inflammatory cytokines, indicating the potential to regulate the extent of inflammation at infection sites due to both Gram-positive and Gram-negative bacteria [105]. It is now known that, in addition to their antimicrobial function, AMPs are potent immunomodulators, playing roles in both pro-inflammatory and anti-inflammatory responses [128, 129]. It was suggested that increased understanding of these multiple functions for OaBac5mini and other AMPs could yield new strategies for the control of inflammatory conditions [105]. For example, it is believed that dysregulated host AMPs are involved in the pathophysiology of a number of autoinflammatory diseases, including psoriasis, atopic dermatitis and rheumatoid arthritis [128, 130].

Thymosin β-4 (Tβ-4), which was first isolated from calf thymus [131], is an anionic peptide that is highly conserved across marine and terrestrial species and is ubiquitously expressed in all tissues and cell types, except erythrocytes [132-134]. In particular, Tβ-4 was identified in human platelets where the peptide had activity against *S. aureus* and *E. coli* that was enhanced by alkaline pH, although the mechanisms underlying this antibacterial activity were not determined [135]. However, human Tβ-4 adopted α-helical structure in membrane mimetic environments [136, 137] and its antibacterial activity appeared to involve interactions between anionic lipid in bacterial membranes and a positively charged region of the peptide, formed by residues 9 to 19 [138]. Similar results were reported for Tβ-4 that serves as an AMP in the sea-urchin, *Paracentrotus lividus* [139], which is highly homologous to its human counterpart and has been predicted to exert its antimicrobial activity using membrane interactive, α-helical structure [140-142].

In addition to antimicrobial activity, it is well established that Tβ-4 is a multifunctional peptide that plays a vital role in wound healing, promoting the repair and regeneration of injured cells and tissues [133, 143, 144]. Given the alkaline pH associated with wounds [21, 145], it has been suggested that the enhanced antimicrobial activity of the peptide under these pH conditions may be an adaptation that allows Tβ-4 to fight microbial infection as a part of the wound healing process [146]. These results strongly support the growing view that platelets are at the nexus of host defence and serve multiple roles in the fight against infection, including the localized release of AMPs and other antimicrobial factors in response to microbial colonization [147-149]. Nonetheless, compared to most AMPs, the antimicrobial activity of platelet Tβ-4 is moderate [135], and a number of recent studies have been suggested that this may not be the primary defence role played by the peptide [150-152]. It was demonstrated *in vitro* that human corneal and conjunctival epithelial cells express Tβ-4 and that the peptide has activity against *P. aeruginosa* [150], which is a common ocular pathogen, [153]. However, human tears strongly inhibited this activity and it was proposed that the major role of the peptide in the eye may be to synergize the activity of AMPs and other antimicrobial factors under inflammatory conditions during microbial infection or epithelial wound healing [150]. Consistent with this proposal, very recent studies showed that the administration of Tβ-4 alone was unable to reduce the bacterial load in a murine model of *P. aeruginosa* induced keratitis [151], which is an infection of the cornea and a leading, global cause of legal blindness [154]. However, when Tβ-4 was administered with ciprofloxacin, the bacterial load and corneal inflammation in this disease model were reduced, whilst corneal wound healing pathways were activated [151]. A follow up to this study showed that Tβ-4 synergizes the activity of ciprofloxacin through indirect antibacterial effects that involve upregulating the production of AMPs in corneal epithelial cells [152]. Based on these

results, it was proposed that Tβ-4 shows promise as an effective, adjunctive therapy to ciprofloxacin in the treatment of bacterial keratitis, offering an alternative to current, standard care regimes [151, 152], which are associated with a number of risks and potential side effects [155, 156]. Currently, there appear to be few other reports in the literature of potential uses for human Tβ-4 based on its antimicrobial capacity, either indirect or direct [133, 143]. However, Tβ-4 a variety of marine creatures have been shown to possess direct antibacterial, antifungal and antiviral action, clearly demonstrating their role as AMPs in the innate immune response of the host and the potential for development as antimicrobial agents [134, 157-159]. For example, derivatives of Tβ-4 from *P. lividus* were shown to be effective against biofilms formed by *P. aeruginosa* [140-142, 160], which are associated with multiple infections that are recalcitrant to conventional antibiotics [161, 162].

The human, salivary mucin 7 (MUC7) plays a primary role in defending the oral cavity from different diseases by reducing bacterial attachment and / or the capacity of these organisms to form biofilms [163-165]. However, MUC7 also appears to contribute to host defence by undergoing specific proteolysis in saliva to yield a variety of peptides [166, 167], some of which have been shown to function as AMPs [167-170]. The best characterised of these AMPs was MUC7 12-mer, which is a cationic peptide formed by residues 40 to 51 of MUC7 [170] and possesses activity against a range of oral bacteria [168] and fungi [171]. A major focus of research into the antifungal activity of MUC7 12-mer has been *Candida albicans* [170, 172-174], which is the major causative agent of oral candidiasis and is becoming increasingly resistant to many conventional, antifungal drugs [175, 176]. MUC7 12-mer possessed potent activity against *C. albicans* in both human saliva and murine models of oral candidiasis that was superior to that of other AMPs and comparable to that of amphotericin B [177, 178], which is a commonly used antifungal drug [179]. More recently, it has been shown that MUC7 12-mer was able to kill *C. albicans* under physiological conditions mimicking those found in the oral cavity and that this ability was enhanced by alkaline conditions [174]. The mechanisms underpinning this antifungal activity were not determined [174] but several genetic studies clearly suggested that this mechanism involved interaction between MUC7 12-mer and the plasma membrane of *C. albicans* [172, 180]. In the first of these studies, it was shown that exposure to MUC7 12-mer upregulated genes in the *C. albicans* calcium / calcineurin signalling pathway, whilst inactivation of this pathway resulted in hypersensitivity to the peptide [172, 181]. It has been previously demonstrated that the calcium / calcineurin pathway is critical to maintaining the integrity of the plasma membrane possessed by *C. albicans* and other Candida species, as well as endowing these organisms with tolerance to antifungals [182-184]. In other studies, genes in the RIM101 signalling pathway were shown to mediate the sensitivity of both *C. albicans* and *Saccharomyces cerevisiae* to MUC7 12-mer, and in both cases, it was suggested that this mediation involved changes to the properties of the plasma membrane [172, 180]. Consistent with this suggestion, the RIM101 pathway has been shown to mediate the sensitivity of *C. albicans* to azoles [185]. which are antifungals that affect plasma membrane integrity [186], through modulating the biosynthesis of sphingolipids [187], which are major components in these plasma membranes [188, 189].

Studies to directly elucidate mechanisms underpinning the action of MUC7 12-mer against *C. albicans* were undertaken, which clearly showed that the peptide adopted α-helical secondary structure with the potential to promote membrane interactions [170]. Consistent with the results of genetic studies [172, 180], the strong positive charge possessed by MUC7 12-mer was shown to promote binding of the *C. albicans* plasma membrane [170]. It is well established that cationic AMPs bind the plasma membrane of fungi *via* interactions with anionic lipid components of these membranes [190]. After initial binding, MUC7 12-mer accumulated on the membrane surface until a threshold concentration was reached, which led to permeabilization of the membrane and uptake of the peptide by cells of *C. albicans*, resulting in the death of the organism [173, 174]. Based on these observations, it was suggested that this antifungal action may show similarities to the two-state model [173, 174], in which AMPs reach a threshold concentration and then realign from a parallel to a perpendicular membrane orientation to form pores [191, 192]. However, the internalization of MUC7 12-mer suggested that the antifungal action of the peptide may involve intracellular sites of action, which was not investigated [173, 174], and the antifungal action of AMPs is well known to include targets such as nucleic acid synthesis and mitochondrial function [181, 193]. Established membrane permeabilizing mechanisms that were also consistent with the reported antifungal action of MUC7 12-mer, would include that described by the SHM model [173, 174].. Essentially, the SHM model incorporates membrane permeabilizing elements of the toroidal pore and carpet mechanisms and includes the formation of transient lesions that facilitate the internalization of AMPs [47, 70, 71].

Based on these results, it was proposed that Tβ-4 shows promise as an effective, adjunctive therapy to ciprofloxacin in the treatment of bacterial keratitis, The major role proposed for MUC7 12-mer was for development to treat oral diseases [174, 177, 178]; for example, a number of studies investigated the potential of its d-amino-acid isomer (MUC7 12-mer-d) to serve in this capacity [168, 177, 178]. It was found that MUC7 12-mer-d showed comparable activity to the native peptide against oral pathogens, both bacterial [168] and fungal [178] but was more resistant to salivary degradation [177]. Based on these investigations, MUC7 12-mer-d was patented for development as an agent in the treatment of diseases such as candidiasis and dental caries [194]. As another example, MUC7 12-mer was studied for the capacity to participate in combination therapy and was shown to synergize the activity of P-113 (PAC-113) [171]. This peptide is a histidine-rich derivative of the human salivary peptide, histatin-5, in clinical trials for the treatment of oral candidiasis and fungal gingivitis [195]. It was proposed that this synergistic combination of AMPs, as well those involving other AMPs and drugs, will not only enhance the efficacy of oral, antifungal therapy but will also reduce the potential for the development of microbial resistance in this therapy [190, 195, 196].

1.2. AMPs from marine sources

The marine environment covers nearly three quarters of the Earth's surface and organisms that inhabit this environment, including bacteria, fish, algae, fungi, sponges, invertebrates and mammals, live in close proximity to the high levels of pathogenic microorganisms that also populate the seas and oceans [197, 198]. In response, marine organisms have developed highly effective immune system that produce a vast array of diverse AMPs, which has promoted the current trend to search for novel antimicrobials in marine ecosystems rather than terrestrial environments [199-202]. However, surprisingly, the only major marine AMPs with alkaline optima so far reported appear to be protamine, which was first shown to possess antimicrobial properties in the 1930s when it was found to inactivate the Vaccinia virus [203]. It is now known that protamine comprises a diverse family of small arginine-rich proteins found in eukaryotic spermatic cells [204, 205] that are particularly abundant in those of fish [206-208], where these peptides are known as salmine in the case of salmon, and clupeine in that of herrings [204, 205]. Enhanced efficacy against a broad range of bacteria under alkaline conditions as been demonstrated for salmine A1 from the salmon, *Salmo salar* [209, 210], and clupeine Z from the herrings, *Clupea pallasii* [211] and *Clupea harengus* [212]. In contrast to many AMPs, protamine is not amphiphilic and lacks secondary structure under these pH conditions, primarily due to the even distribution of numerous arginine residues along its backbone (Table 2) [204, 213]. These residues also render protamine as one of the most cationic AMPs known [204, 205] and its improved antibacterial efficacy at higher pH appeared to be based on an increased electrostatic affinity for anionic components of membranes [212]. These enhanced electrostatic interactions induced the permeabilization of membranes from Gram-positive bacteria, such as *Listeria monocytogenes*, and Gram-negative organisms, such as *Shewanella putrefaciens* [210, 214]. However, currently, the conformational preferences and mode of lipid interaction involved in the membranolytic, antibacterial mechanisms of the peptide are unknown [212]. In contrast, high levels of electrostatic interaction between protamine and anionic sidechains of OM LPS appeared to inhibit the lytic action of the peptide against some Gram-negative bacteria, including *E. coli* and *P. aeruginosa* [215]. These high affinity interactions appeared to, effectively, bind protamine to the OM surface, and more recent investigations have suggested that protamine kills these bacteria using non-membranolytic mechanisms that involve internalization of the peptide to attack intracellular targets [216, 217]. Strongly supporting this suggestion, protamine is known to have a high affinity for DNA [204, 218, 219], and when directed against *P. aeruginosa*, the peptide caused no damage to membranes of the organism but accumulated intracellularly, inducing an enlarged periplasmic space and condensation of the cytoplasm [220]. Protamine appeared to cross the outer and inner membranes of *E. coli*, *P. aeruginosa* and *S. typhimurium* using mechanisms that were independent of the bilayer, which led to the proposal that the peptide crossed the cell envelope of these bacteria using protein mediated processes [216, 217]. It was suggested that protamine may traverse the OM of *E. coli*, *P. aeruginosa* and *S. typhimurium* using porins [217], which form channels allowing the passive diffusion of small hydrophilic molecules across this membrane [221]. The protein mediated processes that render protamine able to cross the inner membrane of these bacteria were unclear [217] but, as described above, a number of bacterial transport systems are known to facilitate this ability for AMPs [34, 35, 104].

Many of the bacteria killed by protamine are foodborne pathogens, [209-212], particularly *L. monocytogenes* which has caused major outbreaks of listeriosis in the last decade [222], and currently, a major use of these AMPs is food preservation [223]. A variety of other antimicrobial applications for protamine have been recently investigated [220, 224] and, in particular, the dental field where the peptide has been shown to both kill oral bacterial pathogens [225, 226]. Protamine was also able to synergize the activity of conventional antimicrobial agents these bacterial pathogens [227], as well as killing both oral bacterial and fungal pathogens when used with dental materials [228, 229].

Marine by-products are a greatly underutilized resource and techniques based on enzymatic hydrolysis have been developed to extract a wide range of bioactive peptides from these materials, including novel antimicrobials, endogenous AMPs and active fragments of these AMPs [230-232]. As a major example, a strongly cationic fragment obtained from the enzymatic hydrolysis of protamine showed comparable or superior activity to the native peptide when directed against oral bacterial and fungal pathogens [225, 228]. Interestingly, more recent studies on this protamine fragment showed that its activity against various species of Candida involved internalization to attack intracellular targets although the mechanisms underpinning this process were not identified [233]. A major marine source of bioactive peptides is crab shells [234], and anionic fractions of low molecular mass peptides with enhanced antibacterial activity under alkaline conditions were recovered from the ground by-products of *Chionoecetes opilio* (The Snow crab) and *Cancer irroratus* (The Atlantic rock crab) [235, 236]. These ground by products were derived from cephalothorax shells, along with hepatopancreas and hemolymph from digestive systems, and included bioactive peptides with potency against *Vibrio vulnificus* and *Vibrio parahaemolyticus* [235, 236], which are well established crab pathogens [237]. Based on the antibacterial profile of these bioactive peptides, it was suggested that active fragments of endogenous AMPs may have been present in the enzymatic hydrolysates obtained from these crustaceans, with the high pH optima of their antibacterial action representing an adaptation to the alkaline pH of seawater [235, 236]. Supporting this suggestion, both untreated and enzymatically hydrolysed hepatopancreas fractions from *C. opilio* were found to contain antibacterial peptides with high levels of homology to known AMPs [238]. Recent studies have also shown that enzymatic hydrolysates of cephalothorax shells, hepatopancreas and hemolymph from *C. opilio* contain antibacterial peptides homologous to known AMPs that are able to reduce biofilm formation on mild steel plates in seawater [239]. It was proposed that these antibacterial peptides could serve as marine antifouling agents [239] and that the use of natural AMPs that had evolved to accommodate the marine environment would minimize the risk of bacterial resistance developing [200, 240]

In a more recent study by Jiang and colleagues (2018), bioactive peptides extracted from the cephalothorax shells of crabs by enzymatic hydrolysis were modified by the Maillard reaction and found to show both enhanced antibacterial and antioxidant activity under alkaline conditions [241], and similar results have been reported for

other crustaceans [242]. Essentially, the Maillard reaction involves the formation of a covalent bond between the amino group of peptides and the terminal, reducing carbonyl group of polysaccharides, which gives the resulting modified peptides enhanced biological activity and proteolytic stability [243]. In the studies of Jiang and colleagues (2018), fractions containing low molecular mass, Maillard modified peptides (MMPs) showed action against Gram-positive and Gram-negative bacteria that was enhanced as pH was increased to alkaline conditions [241]. It is well established that AMPs and bioactive peptides derived from crustaceans show the potential to serve as natural preservative in food and food products [244] and on this basis, it is proposed that the pH dependent MMPs described above should be investigated for their ability to serve as antimicrobials within this context [241]. Interestingly, these MMPs also showed antioxidant action that was enhanced as pH was increased to alkaline conditions, which could, at least in part, reflect the presence of endogenous antioxidant peptides that have adapted to the alkalinity of marine conditions [241]. Antioxidants play an important role in protecting marine organisms from oxidative stress [245, 246] and endogenous antioxidant peptides, such as glutathione, carnosine, anserine and balenine, have been identified in a variety of these organisms, including fish, seals, whales and dolphins [247-249]. In addition, uncharacterized endogenous antioxidant peptides have been reported in *Ocypoda macrocera* (The red ghost crab) [250], *Actinopyga lecanora* (The stone fish or sea cucumber) [251], *Galatea paradoxa* (The common galatea clam) and *Patella rustica* (The rustic limpet) [252]. Currently, antioxidant peptides, both those endogenous those and obtained from marine by-products, are either in use or show potential for use as dietary supplements, components of skin care products and therapeutics against oxidative damage-related pathological conditions, such as heart disease and strokes [253-255].

1.3. AMPs from amphibians

Amphibians have played a central role in the discovery of AMPs [41], with work on *Xenopus laevis* (The African clawed frog) leading to the identification of magainin in 1987 [256] and the PYL peptide in 1997 [257]. These molecules were amongst the first cationic and anionic AMPs, respectively, to be characterised in detail and, along with other amphibian peptides, work on these AMPs played a major role in the recognition and understanding of the defence and other functions played AMPs in the innate immune system [41, 258]. Amphibians have also been the richest source of AMPs, with over 1000 of these peptides registered in the APD3 database, representing *circa* one third of the total entries for AMPs [258, 259]. Amongst these AMPs are a number with pH dependent activity [37], including several with alkaline optima that have been mainly reported over the last decade or so (Table 1). For example, fallaxin is a cationic, peptide that was first isolated from *Leptodactylus fallax* (The mountain chicken frog) and shows activity against Gram-positive bacteria but is ineffective against Gram-negative organisms and fungi [260]. More recent studies have shown that substituting a leucine residue for alanine at position 9 of fallaxin (Table) generated a cationic, α-helical homologue, FL9, that was able to kill Gram-positive bacteria with a mode of action that was enhanced under high pH conditions [261, 262]. Electrostatic interactions between FL9 and anionic components of the membranes possessed by these organisms were essential to the membranolytic action of the peptide, which appeared to involve a dual mode of action that included membrane lysis and translocation to inhibit DNA synthesis [261, 262]. More recent theoretical studies on FL9 have suggested that these mechanisms may also involve the formation of tilted structure by the peptide [263], which is α-helical architecture that characterized by a hydrophobicity gradient along its long axis [77, 264]. Possession of this form of secondary structure causes AMPs to penetrate membranes at a shallow angle of between 20° and 80°, thereby promoting a range of membrane destabilizing effects that could potentially support membrane lysis and translocation by FL9 [77, 264].

Table 1. Major AMPs with alkaline optima for antimicrobial activity

AMPs	Net charge	Structure	Host organism	References
Mammals				
Thymus peptide	Cationic	Unknown	Calves and sheep	42, 43
SMAP29	Cationic	α-helical	Sheep	96
OaBac5	Cationic	polyproline helices	Sheep	96
OaBac5mini,	Cationic	polyproline helices	Sheep	96
MCP-1	Cationic	α-defensin	Rabbits	63
MCP-2	Cationic	α-defensin	Rabbits	63
NP1	Cationic	α-defensin	Rabbits	65, 67, 75
NP2	Cationic	α-defensin	Rabbits	65, 67
HNP-1	Cationic	α-defensin	Humans	75
Tβ-4	Anionic	Unknown	Humans	135
MUC7 12-mer	Cationic	α-helical	Humans	174
Marine sources				
Salmine A1	Cationic	Unknown	*Salmo salar*	209, 210
Clupeine Z	Cationic	Unknown	*Clupea harengus*	212
Clupeine Z	Cationic	Unknown	*Clupea pallasii*	211
Hydrolysates	Anionic	Unknown	*Chionoecetes opilio*	235
Hydrolysates	Anionic	Unknown	*Cancer irroratus*	236
Hydrolysates	Unknown	Unknown	Crabs	241

Amphibians				
Dy2	Cationic	α-helical	*Rana dybowskii*	[265]
AWRK6	Cationic	α-helical	*Rana dybowskii*	[265]
FL9	Cationic	α-helical	*Leptodactylus fallax*	[261, 262]
E2EM-lin	Cationic	α-helical	*Glandirana emeljanovi*	[266]
Designed AMPs				
ARYV	Cationic	β-sheet	*De novo*	[267, 268]
VDVY	Cationic	β-sheet	*De novo*	[267, 268]
*ARVA	Cationic	β-sheet	*De novo*	[267, 268]
C(12)K-7α(8)	Cationic	Unknown	Synthetic	[269]
$C_{16(\omega7)}K\text{-}\beta_{12}$	Cationic	Unknown	Synthetic	[270]

FL9 showed efficacy against clinically relevant, pathogens, and, in particular, methicillin-resistant *Staphylococcus aureus* [261, 262], which can cause a wide range of infections, ranging from pneumonia to toxic shock syndrome, which caused by the release of bacterial toxins [271, 272]. MRSA is able to express an extensive arsenal of virulence factors and possesses resistance to most classes of antibiotics, clearly necessitating the development of new drugs with novel mechanisms of action to combat infections caused by the organism, which are now endemic in many parts of the world [273, 274]. Undesirably, FL9 showed the potential to induce the expression of virulence genes by *S. aureus*, clearly rendering it unsuitable for uses such as the treatment of *S. aureus* infections or food preservation [261]. However, based on its high pH optimum and tolerance of other environmental conditions, it was proposed that a modified form of FL9 might be suitable for development as a food additive [261].

Dybowskin2- CDYa (Dy2) is a strongly cationic peptide that was originally isolated from the frog, *Rana dybowskii* (Dybowski's frog) and exhibited potent activity against Gram positive and Gram-negative bacteria [275]. This peptide was used to generate the cationic homologue, AWRK6, by the substitution of lysine residues for each of the six arginines possessed by Dy2, as well as the substitution of tryptophan residue for alanine at position 2 of Dy2 (Table 2). In the case of both peptides, alkaline pH promoted increased levels of α-helical structure in the presence of membranes that correlated with enhanced antibacterial activity [265]. Previous studies have shown that pH induced increases in the α-helicity of AMPs can enhance their amphiphilicity and / or hydrophobicity, thereby facilitating higher levels of membranolytic action [37]. Alkaline pH also appeared to reduce the effective positive charge of both Dy2 and AWRK6, suggesting that increased hydrophobicity may play a role in enhancing the membranolytic activity of these peptides at high pH [265]. Strongly supporting membranolytic action for AWRK6, transmission electron microscopy showed that the peptide induced rupture and damage to membranes of *S. aureus* that appeared to be accompanied by the leakage of cytoplasmic contents [265]. However, AWRK6 showed a much higher efficacy against the bacteria studied and a much improved resistance to proteolytic degradation than Dy2 under corresponding pH conditions [265]. Based on these observations, it was suggested that AWRK6 showed the potential for further development as a novel antibiotic [265]; proteolytic susceptibility is a major problem in the development of AMPs for therapeutic use, particularly systemic application [276, 277]. Another potential application proposed for AWRK6 was the treatment of diabetes [278, 279], which is a metabolic disease characterized by elevated levels of blood glucose and a leading cause of mortality and morbidity, worldwide [280, 281]. AWRK6 has also been shown to have potential for combatting inflammatory responses induced by LPS [282], which can lead to endotoxemia and sepsis, a life-threatening syndrome with increasing global incidence [283, 284].

Table 2. Sequences of major AMPs with alkaline optima for antimicrobial activity

AMPs	Sequences of AMPs	Refs
Mammals		
Thymus peptide	Unknown	[43]
SMAP29	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH_2	[96]
OaBac5mini,	RFRPPIRRPPIRPPFRPPFRPPVR-NH_2	[96]
OaBac7.5mini	RRIPRPILLPWRPPRPIPRPQPQPIPRWL	[96]
MCP-1	VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR	[62]
MCP-2	VVCACRRALCLPLERRAGFCRIRGRIHPLCCRR	[62]
NP1	VVCACRRALCLPRERRAGFCRIRGRIHPLCCRR	[65]
NP2	VVCACRRALCLPLERRAGFCRIRGRIHPLCCRR	[65]
HNP-1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	[74]

Tβ-4	SDKPDMAEIEKFDKS KLKKTETQEKNPLPSK ETIEQEKQS	[285]
MUC7 12-mer	RKSYKCLHKRCR	[174]
Marine sources		
Salmine A1from *Salmo salar*,	PRRRRSSSRPVRRRRR PRVSRRRRRRGGRRR R	[219]
Clupeine Z from *Clupea pallasii*	ARRRRSRRASRPVRR RRPRRVSRRRRARRR R	[286]
Clupeine Z from *Clupea harengus*	ARRRRSRRASRPVRR RRPRRVSRRRRARRR R	[286]
Hydrolysates from *C. opilio*	Unknown	[235]
Hydrolysates from *C. irroratus*	Unknown	[236]
Hydrolysates from crabs	Unknown	[241]
Amphibians		
Dy2	SAVGRHGRRFGLRKH RKH	[265]
AWRK6	SWVGKHGKKFGLKK HKKH	[265]
FL9	GVVDILKGLAKDIAG HLASKVMNKL-NH_2	[261]
E2EM-lin	GILDTLKQFAKGVGK DLVKGAAQGVLSTVS CKLAKTC	[287]
Designed AMPs		
ARYV	WALRLYLVYNH_2	[267]
VDVY	RRGWVLDLVLYYGR RNH_2	[267]
*ARVA	RRGWALRLVLAYNH_2	[267]
C(12)K-7α(8)	C12K is dodecanoyl-lysyl and α8 is an aminooctanoyl-lysyl subunit.	[269]
C $_{16(\omega7)}$K-β_{12}	C $_{16(\omega7)}$K is hexadecenoyl-lysyl and β_{12} is a lysyl-aminododecanoyl-lysyl-amide subunit	[270]

Esculentin-2 EM is a cationic, α-helical peptide that was originally isolated from the skin secretions of *Glandirana emeljanovi* (The Imienpo Station frog) [288], and more recently, the linearized form of the peptide (E2EM-lin) was shown to possess potent membranolytic activity against Gram-positive bacteria [289, 290]. The underlying mechanism in this action appeared to be the ability of phosphatidylglycerol, which is the major anionic lipid in the membranes of Gram-positive bacteria [33, 77], to drive the formation of α-helical structure in the N-terminal region of E2EM-lin that included a tilted peptide [290]. Similar conformational changes also underpinned the weaker action of E2EM-lin against Gram-negative bacteria, although this action was driven by phosphatidylethanolamine [290], which is the predominant lipid in the membranes of these organisms [33, 77]. In both cases, alkaline pH enhanced the levels of this lipid induced, N-terminal secondary structure and thereby, the ability of E2EM-lin to induce the lysis of bacterial membranes [263, 266]. These pH conditions were also proposed to enhance the membranolytic activity of E2EM-lin by increasing the hydrophobicity of the peptide through reducing its effective positive charge [263, 266], as described above for Dy2 and AWRK6 [265]. These pH dependent structure / function relationships were used to update a model previously presented for the antibacterial action of E2EM-lin [263, 266], which essentially proposed that the membranolytic mechanisms used by peptide to kill these organisms involved pore formation [289, 291]. According to this updated model, a short, α-helical region at the C-terminus of E2EM-lin, which is demarcated by a glycine residues at position 24 of the peptide, lies on the membrane surface, anchoring the peptide [266, 290]. In this respect, the C-terminal region of E2EM-lin showed similarities to that of *E. coli* penicillin-binding protein 5, which also forms α-helical structure and serves as a membrane anchor for the parent protein [292]. The conformational flexibility provided by the glycine residues at position 24 of E2EM-lin then allows the long N-terminal, tilted region of the peptide to realign and adopt a transmembrane orientation, which leads to the association of these transmembrane regions and the formation of a pore [266, 290]. It is established that as well as promoting membrane destabilizing effects that lead to pore formation, the tilted structure possessed by AMPs can also promote peptide – lipid and peptide – peptide interactions that directly assist in the assembly and stabilization of these structures [77, 264, 293]. Indeed, it was proposed that alkaline pH mediated decreases in the effective positive charge of E2EM-lin may also enhance pore formation by reducing repulsive electrostatic interactions between molecules of the peptide involved in the construction of these structures [266, 290]. Currently, it is

believed that the membranolytic, antibacterial action of E2EM-lin involves the ability of the peptide to form either barrel-stave pores or toroidal pores [289, 290], although the latter pore type appears to be the most consistent with experimental data [289]. The major difference between these two pore forming mechanisms is that with toroidal pores, the membrane leaflets deform to allow the lipid head-group region to remain in contact with the hydrophilic face of the E2EM-lin membrane spanning region, which is not observed with barrel-stave pores [33, 77] In both cases, it has been proposed that increasing pH promotes the ability of E2EM-lin to induce the lysis of bacterial membranes, which is maximal under alkaline conditions [263, 266].

Figure 1. The interactions of E2EM-lin with membranes of *Saccharomyces cerevisiae*

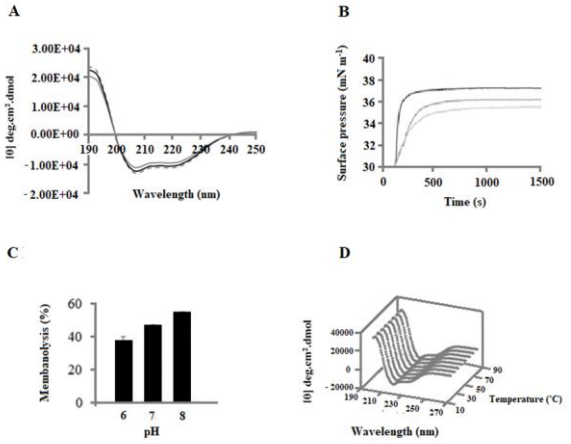

Figure 1 characterizes the interactions of E2EM-lin with membranes formed from native lipid extracts taken from *S. cerevisiae*. Figure 1A shows CD spectra for E2EM-lin in the presence of these membranes, which possess minima in the range 208 nm to 224 nm and a maximum at 193 nm, which is typical of α-helical structure. Analysis of these spectra indicated that these membranes induced conformational changes in the peptide, with levels of α-helicity increasing from 38.0 % at pH 6 (dotted grey line) to 55.0% at pH 8 (grey line). Figures 1B and 1C show that E2EM-lin was also able to interact with membranes derived from *S. cerevisiae*. At pH 6, the peptide induced maximal surface pressures of 5.3 mN m^{-1} in these membranes (light grey line) that increased to 7.3 mN m^{-1} at pH 8 (black line), indicating high levels of insertion (Figure 1B). E2EM-lin was also able to permeabilize these *S. cerevisiae* membranes, promoting 31.0 % lysis at pH 6 that increased to 56.0% at pH 8, indicating strong membranolytic ability (Figure 1C). Figure 1D shows CD spectra across the temperature range, 20˚C to 90˚C, for the peptide in the presence of *S. cerevisiae* membranes, which possess minima in the range 208 nm to 225 nm and a maximum at 193 nm. Analysis of these spectra showed that E2EM-lin possessed α-helical content of *circa* 45%, which remained stable across the temperature range studied. E2EM-lin was found to possess an MIC of 60 μM when directed against *S. cerevisiae*, which, along with the data shown in Figures 1A, 1B and 1C, obtained using previously published methodologies [294].

It has been shown that E2EM-lin is thermostable in the presence of bacterial membranes and based on its alkaline optimum for antibacterial activity, it was proposed that the peptide may be suitable for development as a preservative in the food industry [263]. However, in addition to bacteria, a variety of yeasts with tolerance to high pH and temperatures are known to act as food spoilage organisms [116, 295], and in the present study, the antifungal activity of E2EM-lin has been investigated (Figure 1). These investigations showed that E2EM-lin killed *Saccharomyces cerevisiae* at levels of *circa* 60 μM, which was comparable to that observed for the peptide when directed against Gram-negative bacteria [266, 290]. These investigations also showed that E2EM-lin adopted levels of lipid interactive α-helical structure in the presence of membranes from *S. cerevisiae* that were enhanced by alkaline conditions (Figure 1A). Correlating with these increases in α-helical structure, the peptide also showed an ability to penetrate and permeabilize *S. cerevisiae* membranes that was enhanced by high pH (Figures 1B and 1C). These correlations parallel those observed for the lytic action of E2EM-lin against bacterial membranes [266, 290], and in combination, these data suggest that the antifungal and antibacterial action of E2EM-lin might use similar mechanisms (Figure 1), which appears to be generally the case for AMPs [33, 77]. E2EM-lin was also found to be thermostable in the presence of *S. cerevisiae* membranes (Figure 1D) and together, these results suggest that the peptide may also be suitable for development as an antifungal agent for use in the food industry [263]. Indeed, currently, yeasts and fungi constitute a global threat, not only to food security, but also by causing plagues, famines, the extinctions of species and human mycoses that are exacerbated by increasing resistance to conventional antifungal drugs [296, 297].

1.4. Synthetic AMPs

Despite their clear therapeutic potential, a number of factors have retarded the development of AMPs and, in particular, *in vivo* stability appears to be the key issue limiting their clinical application both in systemic administration and topical use [277, 298]. In response, numerous strategies have been instigated to extend the half- life of AMPs, reduce their cytotoxicity and improve their selective antimicrobial activity, thereby translating these peptides into successful clinical products [47, 299]. In general, these strategies can be divided into two broad categories, the modification of naturally occurring AMPs [300, 301] and the development of *de novo* molecules, in the form of both peptides [302, 303] and synthetic variants and mimics of peptides [304, 305].

In relation to the development of *de novo* AMPs, ARYV, *VDVY* and *ARVA are derived from a designed combinatorial library and are cationic, β-sheet peptides with the ability to bind and permeabilise model bacterial membranes [306]. ARYV, *VDVY* and *ARVA showed potent activity against *S. aureus* that was enhanced under alkaline conditions and attributed, in part, to higher levels of negative charge on teichoic acids of the *S. aureus* peptidoglycan layer promoting increased interaction with these peptides [267, 268]. Based on these results, it was proposed that manipulating pH could improve the efficacy of these AMPs as anti-biofilm peptides in a clinical setting [267, 268], which would appear to be supported by recent studies [307]. Increased negative charge on teichoic acids under high pH conditions were found to arrest the development of *S. aureus* biofilms, which led to the suggestion that alkaline

formulations including cationic antimicrobials could be used to reduce the risk posed by these biofilms [307], known to be a leading cause of nosocomial infections [308].

With respect to synthetic mimics of AMPs, a particularly promising group of antimicrobial molecules are peptidomimetics based on the sequences of AMPs isolated from Hylid frogs [309], namely dermaseptins [310]. These peptidomimetics are constructed from alternating acyl chains, usually a fatty acid of variable length, and cationic amino acids, predominantly lysine, and are generally referred to as oligoacyllysines or OAKs [309]. These peptidomimetics have broad range antimicrobial activity [304, 311] and several OAKs, C(12)K-7α(8) and $C_{16(\omega7)}$K-β_{12}, have been shown to exhibit antibacterial action that is enhanced by high pH [269, 270]. Target bacteria, included MRSA, *L monocytogenes* Li2 and *E. coli* O157:H7, and in all cases, the activity of these OAKs was more potent than that of ciprofloxacin [269, 270], which is a fluoroquinolone with optimum efficacy at high pH [25, 26]. C(12)K-7α(8) exhibited an ability to bind bacterial membranes that was enhanced by high pH [269] and taken with the results of previous studies [312, 313], clearly suggested that the peptidomimetic utilized membranolytic, antibacterial mechanisms with alkaline optima [269]. In the case of $C_{16(\omega7)}$K-β_{12}, the peptidomimetic also appeared to kill bacteria using membranolytic mechanisms [270], which was supported by studies showing that $C_{16(\omega7)}$K-β_{12} was able to promote the uptake of intracellular acting antibiotics by *E. coli* and other Gram-negative bacteria [314]. Interestingly, $C_{16(\omega7)}$K-β_{12} appeared to kill one strain of *E. coli* using a non-membranolytic, intracellular mode of action that involved attack on DNA, which was proposed to the first report of a given antimicrobial using differing mechanisms to kill strains of the same organism [270]. The mechanisms underpinning this effect were not investigated but differences between *E. coli* strains are well known; for example the expression of porins [315, 316], which, as described above, appear to mediate the uptake and intracellular action of protamine against Gram-negative bacteria [217, 220]. Based on these studies, a variety of applications were suggested for both C(12)K-7α(8) and $C_{16(\omega7)}$K-β_{12}, and in particular, reflecting their high efficacy against *L. monocytogenes*, development as antimicrobials in food safety and food products [269, 270].

2. DISCUSSION

It is becoming increasingly clear that AMPs with pH dependent antimicrobial activity are produced by creatures across the eukaryotic kingdom and most of these peptides possess acid optima, which has led to a range of therapeutic and biotechnical applications [37]. However, this review has shown that a growing number of AMPs with alkaline optima for their antimicrobial action are also produced by creatures across the eukaryotic kingdom, including humans, rabbits, cattle, sheep, fish and frogs (Table 1). AMPs with a similar pH dependency have also been generated by the enzymatic hydrolysis of marine by products, and designed, *de novo*, in the form of both peptides and synthetic variants and mimics of peptides (Table 1). Most of these AMPs were cationic, with the exception of Tβ-4 [135] and peptides derived from the by-products of *C. opilo* and *C. irroratus* [235], which currently, would appear to be the only major, known example of anionic AMPs with alkaline optima. Indeed, in relation to cationic AMPs, anionic AMPs are relatively rare and possess lower efficacy, which led to the suggestion that they may have evolved to synergize and broaden the spectrum of activity shown by cationic AMPs [317, 318]. Consistent with this suggestion, Tβ-4 formed part of a suite of pH dependent platelet AMPs, both cationic and anionic, with overlapping antimicrobial spectra [135], and platelets play key roles in host defence against infection by a diverse range of microbial pathogens, including bacteria, fungi, viruses and protozoa [147-149].

The AMPs reviewed here showed antiviral, antibacterial, antifungal and antioxidant activity that involved membrane interactions and appeared to be facilitated by a variety of mechanisms to target these membranes. In the case of anionic AMPs, there exists the potential for repulsive electrostatic interactions when targeting microbial membranes and to overcome these interactions, these peptides utilise a range of strategies [317, 318]. Tβ-4 is strongly anionic under alkaline conditions [135] and appears to utilise one of the most frequently used of these strategies, which is to target microbial membranes *via* a cationic region of its sequence, effectively functioning as a positively charged AMP [138]. In cases, where no such cationic regions exist, anionic AMPs often use the charge neutralizing effects of divalent metal ions to facilitate interaction with microbial membranes [317, 318]. For example, peptides primarily composed of aspartic acid residues have very recently been shown to form complexes with zinc ions to target and facilitate the disruption of bacterial membranes *via* pore formation [319].

In relation to the cationic AMPs, protamine, Dy2, AWRK6 and E2EM-lin, although alkaline pH reduced their net positive charge, it enhanced their electrostatic interactions with microbial membranes and their antimicrobial activity [263, 265, 266]. These observations contrast strongly to most cationic AMPs where a reduced positive net charge under these pH conditions generally leads to the diminution or abolishment of this activity [36, 37]. It has been suggested that the decreasing the net positive charge of the AMPs reviewed here effectively increases their hydrophobicity, thereby enhancing their membrane interactive potential and antimicrobial action [263]. However, these observations also clearly suggest that some other factor(s) help mediate the strength of electrostatic interaction with membranes exhibited by protamine, Dy2, AWRK6 and E2EM-lin, and a clear candidate would appear to be the level of anionic charge carried by their target membranes. This suggestion appears to receive support from studies on E2EM-lin, which showed that the ability of alkaline conditions to enhance the interactions of the peptide with bacterial plasma membranes was modulated by the composition of these membranes [263, 266]. The major drivers of these membrane interactions were PE and PG in the cases of Gram-positive and Gram-negative bacteria respectively, but the net charge carried by these lipids appeared to be unaffected by changes in pH [320, 321]. However, the electrostatic interactions of E2EM-lin with membranes from both of these bacterial types also appeared to involve contributions from cardiolipin [263, 266], which is a common anionic component of these membranes that is known to become more anionic under alkaline conditions [322, 323]. The role of CL in the antimicrobial action of E2EM-lin was unclear but, based on these observations, one function of the

lipid may be the enhancement of electrostatic interaction between E2EM-lin and microbial membranes at higher pH, thereby helping to compensate for the decreased net charge of the peptide. In relation to protamine, alkaline conditions enhanced the electrostatic interactions of the peptide with the OM of some Gram-negative bacteria, which promoted membranolytic mechanisms [210, 212, 214]. In this case, it is well established that LPS carries multiple side chains whose level of negative charge is enhanced by higher pH, suggesting that the lipid may serve a role similar to that proposed above for CL; namely, the enhancement of electrostatic interaction between protamine and the OM under alkaline conditions [324, 325]. However, with other Gram-negative bacteria, electrostatic interaction between protamine and the OM under alkaline conditions appeared to inhibit the ability of E2EM-lin to engage in membranolytic mechanisms [215] but not its ability to cross this membrane *via* diffusion down porin channels [216, 217, 220]. In this case, it may be that alkaline conditions could enhance the overall affinity and binding of protamine to the OM such that the peptide was inhibited from inducing membranolytic mechanisms but was sufficiently mobile to locate and interact with porins [216, 217, 220]. In combination, these results suggest that decreased net positive charge under alkaline conditions could be considered to constitute a structure / function relationship that helps optimise the membrane affinity required by protamine, E2EM-lin and, most likely, other AMPs reviewed here, to facilitate their antimicrobial action. These combined results also strongly reinforce the view that, to provide a full description of the antimicrobial and biological activity of AMPs, it is essential that the characteristics of both these peptides and their target membranes are taken into account [326, 327].

Apart from protamine [216, 217, 220], most of the peptides reviewed here with action against Gram-negative bacteria appeared to translocate the OM using lipid mediated processes, which in the case of OaBac5mini, OaBac7.5mini and SMAP-29 (SMAP-28) was reported to involve the self-promoted pathway uptake mechanism [91, 99, 100, 105]. Upon gaining access to the inner membranes of Gram-negative bacteria, and the plasma membranes of Gram-positive bacteria, the AMPs reviewed here exerted their antibacterial action using a variety of mechanisms. Several AMPs appeared to use non-lipid mediated mechanisms, including protamine, which was predicted to employ unidentified protein pores [216, 217], as well as OaBac5mini and OaBac7.5mini, which were proposed to utilise bacterial transporters, possibly Sbma and MdtM [34, 104, 112, 113]. The use of bacterial transporters to gain cell entry is increasingly being reported for proline-rich AMPs [34, 104, 112, 113], such asTur1A from dolphins (*Tursiops truncates*) [112], but is generally rare, with the only other major examples, including some prokaryotic AMPs [104] and human antifungal peptides [328, 329]. In the case of protamine, OaBac5mini and OaBac7.5mini, the conformational flexibility of these strongly charged peptides appeared to be important to facilitating both their protein mediated uptake and their ability to attack internal targets, including DNA and protein synthesis [99, 105, 216, 217]. For example, it is believed that proline-rich AMPs, such as OaBac5mini and OaBac7.5mini, inhibit bacterial protein synthesis by interacting with the large subunit of the ribosome and specifically binding within the ribosomal exit tunnel. Within this tunnel, proline-rich AMPs adopt an elongated conformation, which includes stretches of polyproline type II helical structure, and effectively blocks transition from the initiation to the elongation phase of protein synthesis [34, 104, 112, 113].

The remaining AMPs reviewed here appeared to use lipid mediated mechanisms to kill microbes and included the α-defensins, HNP-1, NP1 and NP2, which were the only reported peptides to have multiple antimicrobial activities that each possessed an alkaline optimum [60, 63, 64, 67-69, 75]. Each of these peptides showed antiviral and antibacterial activity that appeared to involve membranolytic mechanisms and were facilitated by electrostatic binding to the viral envelope [60, 63, 64] and bacterial membrane, respectively [67-69, 75]. These AMPs also differed to the other peptides reviewed here in that α-defensins possess conformationally restrained molecules due to their cysteine stabilized structures and interact with membranes *via* tertiary amphiphilicity [57, 66]. In relation to their antibacterial activity, HNP-1, NP1 and NP-2, showed activity against Gram-negative bacteria and Gram-positive bacteria using membranolytic mechanisms that showed similarities to the toroidal pore mechanism in the case of the former bacteria [57, 65, 67, 75]. The toroidal pore model is the most cited pore mechanism for membrane by AMPs [33, 72, 77] and as a historical note was initially proposed to describe the antibacterial action of magainins from *X. laevis*, which, as described above, was one of the first AMPs to be identified [41]. With regards to the antiviral activity of HNP-1, NP1 and NP-2, this work was the first to report that α-defensins were able to inactivate viruses [330], and initially, it was believed that envelope permeabilization was the major mechanism used by these AMPs to kill these microbes [64]. However, it is becoming increasingly clear that this family of AMPs uses multiple mechanisms to inactivate enveloped viruses that that are not membrane associated, as well as possessing the ability to inactivate nonenveloped viruses [64, 331].

In contrast to the α-defensins described above, SMAP-29 (SMAP-28), Tβ-4, MUC7 12-mer, Dy2, AWRK6, FL9 and E2EM-lin, appeared to exert their membranolytic and antimicrobial action through the adoption of amphiphilic, α-helical structure. In the case of Dy2, AWRK6 and E2EM-lin, the ability of alkaline pH to promote higher levels of this α-helical structure was shown to underpin their enhanced membranolytic and antimicrobial activity under these pH conditions [265, 266], and it seems possible that similar structure / function relationships could contribute to the biological activity of other α-helical AMPs reviewed here (Table 2). MUC7 12-mer and E2EM-lin were found to possess activity against yeasts and fungi that was enhanced by alkaline pH, which would appear to be the first major report of antifungal AMPs exhibiting this form of pH dependency, although antifungal peptides with acid optima have been reported [332, 333]. The antifungal action of MUC7 12-mer showed similarities to the SHM model of membrane permeabilization [173, 174], and it was suggested that the antifungal activity of E2EM-lin may use a toroidal pore type model, based on similarities to its antibacterial action (Figure 1). As described above, it is recognised that AMPs generally use similar membrane permeabilising mechanisms across their biological activities [33, 77], although clear differences can exist between antifungal and antibacterial peptides [334, 335]. For example, some plant AMPs with antifungal activity are known to utilise fungal lipids as receptors to promote

internalization, which induces signalling cascades and interaction with intracellular targets, thereby promoting the formation of reactive oxygen species that ultimately leads to apoptosis [334, 335]. The use of receptors in the antibacterial mechanisms of AMPs is known but rare [336-338], and in contexts such as the induction of apoptosis, the action of antifungal peptides shows commonalities with the anticancer action of AMPs, as may be expected, given the eukaryotic nature of both fungal and cancer cells [326, 339].

The amphiphilic, α-helical structure adopted by SMAP-29 (SMAP-28), Dy2, AWRK6, FL9 and E2EM-lin under appeared to promote membranolytic mechanisms that led directly to the death of target bacteria. SMAP-29 (SMAP-28) and E2EM-lin are the best characterized of these AMPs and appear to use the carpet and toroidal pore mechanisms of membrane perturbation, respectively. More recently, it has been suggested by a computational study based on the activity determinants of α-helical AMPs that E2EM-lin may utilize a carpet type mechanism in its antimicrobial action [340]; although in some cases, this mechanism can be considered as multiple toroidal pore formation [77]. In the case of FL9 and E2EM-lin, the amphiphilic, α-helical structure adopted by these peptides appeared to include tilted architecture [263, 266]. This architecture is a novel structure / function relationship that has been shown to promote the membranolytic and antimicrobial activity of other amphibian AMPs [263, 341, 342], including those with pH dependent action [343], and has the potential to serve a similar role in the activity of many other of these peptides [263, 344]. In the case of E2EM-lin it has previously been suggested that a number of lysine residues located in the N-terminal region of the peptide may help promote the membrane orientation of its tilted structure by use of the snorkelling mechanism [290]. According to this mechanism, the α-carbons of lysine residues are able to reside in the membrane core region whilst their long alkyl side-chains extend, allowing the positively charged moieties of these residues to engage in electrostatic interactions with the lipid headgroup region [345]. Lysine residues carry a reduced net positive charge under alkaline conditions and it would seem that the resulting increase in local hydrophobicity could potentially enhance the ability of tilted structure in E2EM-lin to snorkel into the membrane, thereby promoting toroidal pore formation [346]. The snorkelling mechanism, which was first proposed over three decades ago to help describe the membrane interactions of apolipoproteins [347, 348], has been reported to play a role in the antimicrobial action of a number of other amphibian AMPs with tilted structure [294, 344, 349]. Interestingly, amphibian peptides that do not form pores or channels have been recently shown to translocate and permeabilize membranes using mechanisms that involve using the snorkelling mechanism to associate with both faces of the membrane and the induction of highly short-lived water bridges [350].

A diverse variety of potential roles in the medical, dental and biotechnical arenas have been proposed for the AMPs reviewed here, including use as antivirals, antibacterials, antifungals, adjuvants to antimicrobial therapy, biomarkers of disease and probes for pathogenic microbes. Based on these results, a number of peptides and peptidomimetics with alkaline optima have been produced with the potential for development as agents in the medical and biotechnical fields. These molecules include the *de novo* AMPs, ARYV, *VDVY* and *ARVA, to act as templates for medically relevant, anti-biofilm agents [267, 268, 306], and the synthetic peptides, C(12)K-7α(8) and $C_{16(\omega 7)}K\text{-}\beta_{12}$, to serve as the basis for antimicrobials used in food products [269, 270]. Peptides with with alkaline optima have also been identified in enzymatic hydrolysates obtained from the shells of a variety of crustacean, which have the potential to act as marine antifouling agents and as preservative in food [235, 236, 241]. Other peptides with alkaline optima derived from this crustacean source showed antioxidant properties [235, 236, 241] that could potentially be developed for a range of dietary, dermatological and therapeutic purposes [253-255]. Although generally beyond the scope of this review, another potential source of AMPs with alkaline optima is alkaliphilic prokaryotes, which has become an established discipline since the seminal work of Horikoshi [351]. These studies showed that many microbes occupy niches that are characterised by an alkaline pH [351], which are now known to range from marine environments, where pH values are typically up to 8.2 [352], and the gut compartments of insects, where pH values up to 12 can exist [353]. Indeed, microbes have also been found in industrial settings, such as sewage plants and soda lakes, where values of pH can exceed 12 [354, 355], and research into alkaliphilic microbes across these various environments has yielded a range of novel chemicals, including enzymes and, in particular, antimicrobial compounds [356-358].

CONCLUSION

In summary, AMPs with alkaline optima represent an untapped source of novel antimicrobials with a wide range of microbial targets and potential applications that is awaiting full exploitation and could help supply the urgent need for alternatives to conventional antibiotics. This review has identified around twenty-five of these peptides, which is a surprisingly low number, given that around 3000 AMPs are listed in the APD database [258, 259]. One reason for this apparent paucity, could be that when characterizing AMPs, it is generally assumed that AMPs have optimum activity under physiological pH conditions [36], which is usually taken as close to neutral pH and are often cited as pH 7.4 [36]. However, although this is a strict pH requirement in cases such as blood [359], physiological pH can also vary considerably [36]. For example, the stomach pH of humans is around pH 1.5 [360], the internal pH of macrophage phagosomes is *circa* pH 5.0 [361] and pH in the urinary tract can normally vary between pH 4.0 and pH 8.0 [362]. In addition, a number of diseases and conditions are associated with pH values out of the physiological range, including those that relate to alkaline pH, as described above, and those that relate to acidic pH [37, 363], such as lung infections in cystic fibrosis [364]. Moreover, it cannot be assumed that the optimal pH for the action of a given AMP is the same for all microbes, as shown by the studies described above on ARYV, *VDVY* and *ARVA: although alkaline pH enhanced their activity against *S. aureus*, it inhibited their action against *P. aeruginosa* and *C. albicans* [267, 268]. It would therefore seem to be crucial that, when characterizing and designing AMPs, account is taken of the pH at both their site of action and during their delivery to this site, which currently does not appear to be generally the case [36, 37]. Indeed, in many earlier studies on AMPs, determining the

effect of pH on their activity across the pH spectrum was a common practice and it is strongly recommended that this practice should be resumed [60, 63, 67, 75].

CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

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