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Antimicrobial peptides in animals and their role in host defences

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Abstract

Domesticated animals have a large variety of antimicrobial peptides that serve as natural innate barriers limiting microbial infection or, in some instances, act as an integral component in response to inflammation or microbial infection. These peptides differ in size, composition, mechanisms of activity and range of antimicrobial specificities. They are expressed in many tissues, polymorphonuclear leukocytes, macrophages and mucosal epithelial cells. There is a small group of anionic antimicrobial peptides found in ruminants and a much larger group of cationic antimicrobial peptides found in all domesticated animals. The cationic peptides include linear, helical peptides, linear peptides rich in proline and cysteine-stabilized peptides with a β -sheet and are commonly referred to as cathelicidins and defensins. These peptides are generally broad-spectrum for Gram-positive bacteria, Gram-negative bacteria and fungi (e.g. myeloid antimicrobial peptides, α -, β -defensins, and protegrins) or are specific to one of these groups (e.g. porcine cecropin P1, Bac5, Bac7, PR-39 and prophenin).

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1. Introduction

The use of low levels of antibiotics as growth promoters in animal feeds [1–3] and the extensive use of antibiotics to treat human or animal infections [4–6] are thought to be the cause of an alarming increase in antibiotic resistance among Gram-negative, Gram-positive and fungal pathogens, a highly debated and controversial issue [7,8]. Bacterial resistance to many classes of antibiotics is becoming a major clinical problem [9,10]. Therefore, the search continues for new antibiotics that are active in vivo, are fast acting and broad-spectrum, do not induce bacterial resistance and have limited side effects. Synthetic congeners of natural antimicrobial peptides are good candidates. In addition to the properties described above, they have low MICs and broad-spectrum activity in both low and

high ionic strength conditions [11], neutralize LPS [12], promote wound healing [13,14], and have synergistic activity with conventional antibiotics [15]. Very few side effects have been reported.

A large variety of antimicrobial peptides are found in domesticated animals (Table 1). They are expressed in many tissues, polymorphonuclear leukocytes, macrophages and mucosal epithelial cells (Table 2). These peptides are broad-spectrum with potent antimicrobial activity against both human and veterinary pathogens. Domesticated animals serve as a valuable source of antimicrobial peptides. They also serve as models to test the efficacy, wound healing effects, and immunological properties of antimicrobial peptides. Antimicrobial peptides are conserved in their structure, function and mechanisms of action, and thus it is attractive to speculate that synthetic antimicrobial peptides or their congeners might be used to prevent or treat infections. As we begin to identify the mechanisms to initiate synthesis and release of antimicrobial peptides, it may also be possible to find ways to trigger their production

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Table 1
Classes of antimicrobial peptides from domestic animals

Class of peptide	General antimicrobial activity (references)
<i>Anionic peptides</i>	
Rich in aspartic and glutamic acids	Gram-negative, Gram-positive bacteria [22,24]
<i>Cationic peptides</i>	
Linear and amphipathic helical molecules	
Seminalplasmin	Broad-spectrum [121]
Myeloid antimicrobial peptides (PMAP, SMAP, BMAP)	Broad-spectrum [11,41]
Porcine cecropin P1	Gram-negative, some Gram-positive bacteria [37,39]
eCATH-1, eCATH-2, eCATH-3	Broad-spectrum [35,50]
Linear peptides rich in proline	
Bac5 and Bac7	Gram-negative bacteria [20] but can be broad spectrum [54]
PR-39	Gram-negative bacteria [20,59]
Indolicidin (rich in tryptophan)	Gram-negative, Gram-positive bacteria [69]
Prophenin (rich in proline and phenylalanine)	Gram-negative bacteria [67]
<i>Cysteine-stabilized sheet molecules</i>	
α -, β -Defensins	Broad-spectrum [20,77,81,122]
Rhesus theta defensin-1 (RTD-1)	Broad-spectrum [94]
Protegrins	Broad-spectrum [41,97]

in animals at stressful times when they are most susceptible to microbial infection (e.g. during transport, castration, weaning, etc.). In this review, an updated list of antimicrobial peptides found in domesticated animals is presented, including sites of antimicrobial peptide production, antimicrobial activity, and their potential use for therapeutic treatment of infectious disease.

2. Types of antimicrobial peptides and sites of antimicrobial expression

Antimicrobial peptides have been a popular topic of research and over 750 eucaryotic antimicrobial peptides have been reported (<http://www.bbcm.univ.trieste.it/~tossi/pag5.htm>). These peptides are grouped according to similarities in charge, sequence homology, functional similarity and 3-dimensional structure [16–20].

2.1. Anionic antimicrobial peptides

In 1992, ovine pulmonary surfactant was reported to kill *Mannheimia haemolytica*, *Escherichia coli* and *Klebsiella pneumoniae* [21]. The antimicrobial activity was due to three small (721.6–823.8 Da) anionic

peptides: H-GDDDDDD-OH, H-DDDDDDDD-OH and H-GADDDDD-OH [22]. These peptides required zinc for maximal activity [22] and were very similar to the charge-neutralizing propeptides of larger zymogens [23], which when synthesized were also antimicrobial [24]. In addition to their innate antimicrobial activity, anionic antimicrobial peptides may also have a regulatory role in pulmonary metabolism. Their structure is similar to the charge-neutralizing propeptides of Group I serine proteases, and they may be capable of regulating, via negative feedback inhibition, the activity of pulmonary enzyme systems. Anionic peptides have been shown to be trypsin inhibitors.

Recently, anionic peptides have also been detected in cattle [25]. Anionic peptide concentration in bronchoalveolar lavage fluid from neonatal calves was found to be about threefold higher than that in adult cattle. However, bronchoalveolar lavage fluid from neonates had significantly less antimicrobial activity against *M. haemolytica* compared with the activity of bronchoalveolar lavage fluid from adults [25].

In Western blots, antibody to anionic peptides detected larger zymogens in solubilized turbinate, tracheal, pulmonary tissue, liver, and small intestine [26]. In pulmonary tissue sections, antibodies identified accumulated protein in the apical cytoplasm of the bronchial and bronchiolar epithelium, in the cytoplasm of pulmonary endothelial cells and in an occasional alveolar macrophage [26].

The mechanism of bacterial killing by anionic peptides is not known. Anionic peptides require zinc for maximal activity [22,27–29] and form a complex with it [30]. Therefore, it is attractive to speculate that zinc may form a cationic salt bridge that allows the peptide to overcome the net negative charge on the microbial surface. The peptide then penetrates the outer membrane without inducing any morphological changes [22]. Once in the cytoplasm, anionic peptides may then attach to ribosomes and inhibit ribonuclease activity similar to that seen with polymers of aspartic acid [31–33]. Ultimately, the cytoplasmic protein precipitates and settles out, suggesting an internal mechanism of protein inactivation (Fig. 1). Killing occurs within 30 min [22].

2.2. Cationic antimicrobial peptides

Cationic antimicrobial peptides are very common in domesticated animals and vary considerably in their composition, structure and distribution among species [18,20]. They occur in three principle categories [16,17,19,20]: linear, helical peptides (Table 3), linear peptides rich in proline (Table 3) and cysteine-stabilized peptides with a β -sheet (Table 4). Within each of these groups are cathelicidins [34,35], peptides that differ greatly in their sequences, structures and sizes. All have a high content of the basic amino acids arginine

Table 2
Antimicrobial proteins in animals

Animal species	Peptide	Tissue/cell type	References
<i>Cattle</i>	Anionic peptides		[25]
	BNBD-1–3, 6–11, 13	neutrophils	[77–80]
	BNBD-4	bone marrow, distal small intestine, trachea, lung, spleen, colon, bovine alveolar macrophages	[78,80]
	BNBD-5	bovine alveolar macrophages	[80]
	BNBD-12	bone marrow, distal small intestine, trachea, colon	[78]
	TAP	nasal epithelium, trachea, bronchioles, alveolar macrophages	[81,82,84]
	LAP	alveolar macrophages, tongue	[80,83]
	EBD	alveolar macrophages, intestine	[80,123]
	BMAP27	bone marrow myeloid cells	[34,124]
	BMAP28	bone marrow myeloid cells	[34,124]
	BMAP34	neutrophils, bone marrow myeloid cells, spleen, testis	[34,49]
	Bac5, Bac7	neutrophils	[34,53,56,125]
	Indolicidin	neutrophils	[34,69–71]
Dodecapeptide	neutrophils	[126,127]	
<i>Sheep</i>	Anionic peptides	turbinates, trachea, pulmonary tissue, liver, small intestine	[22,26]
	Sheep BD-1	tongue, trachea, rumen, reticulum, omasum, colon	[86]
	Sheep BD-2	ileum, colon	[86]
	SMAP28	neutrophils	[45,46]
	SMAP29	bone marrow myeloid cells	[11,43–46]
	SMAP34	bone marrow myeloid cells	[11,43–46]
	OaBac5 α , β	neutrophils	[54]
	OaBac6	sheep genomic library	[128]
	OaBac7.5	bone marrow myeloid cells	[44,128]
	OaBac11	sheep genomic library	[128]
Dodecapeptide	bone marrow myeloid cells	[44]	
<i>Goats</i>	Goat BD-1	tongue, respiratory tract	[88]
	Goat BD-2	intestine	[88]
	ChBac5	neutrophils	[54]
<i>Pigs</i>	Porcine BD-1	respiratory tract, digestive tract, thymus, spleen, lymph node, brain, liver, kidney, urinary bladder, testis, skin, heart, muscle, bone marrow, neutrophils, alveolar macrophages, umbilical cord	[129]
	PMAP23	bone marrow myeloid cells	[40,42,60]
	PMAP36	bone marrow myeloid cells	[40,41,130]
	PMAP37	bone marrow myeloid cells	[60,131]
	PR-39	intestinal epithelium, neutrophils	[38,58,62,132]
	β PR-39	porcine liver genomic library	[60]
	Cecropin P1	small intestine	[38,39,58]
	Prophenin-1, 2	leukocytes	[60,67,68,133]
	Protegrin 1-5	leukocytes	[60,97,134]
<i>Horses</i>	eNAP-1	neutrophils	[135]
	eCATH-1, eCATH-2, eCATH-3	neutrophils	[35,50]
<i>Poultry</i>	Gal 1/CHP1	heterophils	[90,91]
	Gal 1 α /CHP2	heterophils	[91]
	Gal 2	heterophils	[90,91]
	Gal 3	tongue, bursa of Fabricius trachea, skin, oesophagus, air sacs, large intestine, kidney	[91,93,136]
	THP 1	heterophils	[90,137]
	THP 2	heterophils	[90,137]
	THP 3	heterophils	[91,93,136,137]
GPV-1	heterophils	[91,93,136]	

and lysine [11,36]. They have a common N-terminal preproregion of about 100 residues that is homologous to the cysteine protease inhibitor cathelin [13]. This conserved region also serves as the target for searches of

new cathelicidins. The highly variable C-terminus contains the cationic antimicrobial domain. After synthesis, the C-terminus is cleaved off, forming the mature antimicrobial peptide.

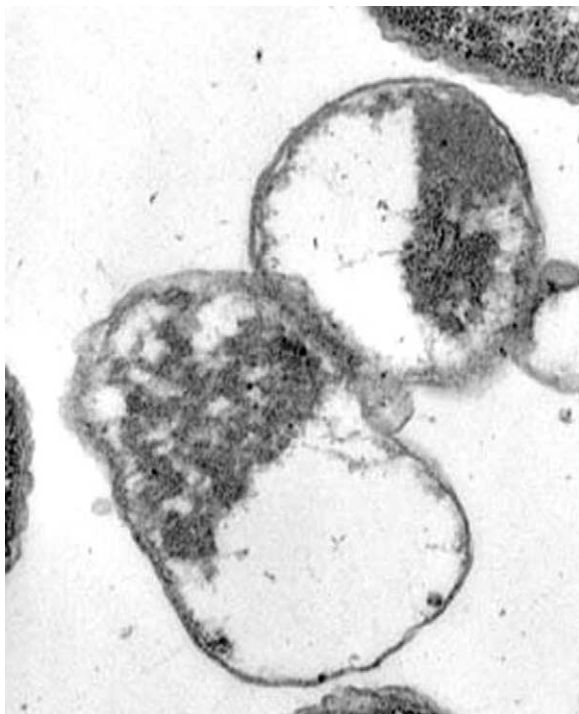


Fig. 1. Transmission electron micrograph of *M. haemolytica* incubated in zinc saline solution containing 0.5 mM anionic peptide. Note the distended outer envelope and flocculated intracellular constituents in affected bacterial cells [22].

2.2.1. Linear and amphipathic peptides that form a α -helix

These peptides are 20–40 residues in size, linear and helical without cysteine residues, and may or may not have a hinge or ‘kink’ in the middle. If they assume a α -helical structure, they become amphiphilic, which is necessary to permeabilize the bacterial cell membrane. Animal cathelicidins in this group include porcine cecropin P1; myeloid antimicrobial peptides from pigs (PMAP), sheep (SMAP) and cattle (BMAP), and the equine cathelicidins eCATH-1, eCATH-2 and eCATH-3 (Table 1).

Cecropins are basic polypeptides with very potent and rapid antibacterial activity [37,38]. Cecropin P1, isolated from porcine small intestine, forms a helical molecule with a hinge-like sequence of Ser-Glu-Gly. It has a strong basic N-terminus and a hydrophobic stretch at the C-terminus and is highly active against Gram-negative bacteria and some Gram-positive bacteria [37–39]. The C-terminus carboxyl group is important for optimal activity against Gram-negative bacteria [38]. P1 is thought to bind to acidic phospholipids in the bacterial membrane and perturb the lipid packing in the bilayers [37]. This mechanism explains the specificity of this peptide for Gram-negative bacteria [37].

A search of porcine bone marrow identified a transcript with an extended N-terminus similar to the cathelin proregions of porcine, bovine and rabbit

defence peptides [40]. The C-terminus encoded a novel peptide termed PMAP23 [40]. This peptide is unique and has only about 35% homology with CAP18 [41]. The sequence is highly cationic with five arginines and two lysines. Synthesized peptide displays remarkable in vitro antimicrobial activity against Gram-negative bacteria [41], Gram-positive bacteria [41], yeasts [42] and moulds [42]. MICs range from 2–16 μ M [40,41]. Antibacterial [41] and antifungal activities [42] appear to be at the plasma membrane. The peptide can also prevent the regeneration of fungal cell walls.

SMAP29 and SMAP34 are potent, antimicrobial peptides of intense interest as potential candidates for the therapeutic treatment of acute and chronic respiratory infections including *Pseudomonas aeruginosa* associated with chronic respiratory inflammation in cystic fibrosis [11,43–47]. The structure and composition of SMAP29 (also known as SC5) was first deduced from sheep myeloid DNA [44,45] and later synthesized to assess its antimicrobial activity [11,43,46]. SMAP29 has broad-spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria and fungi [11,43,45,46], is active in both low and high ionic strength conditions [11], and induces significant morphological alterations in bacterial surfaces [46,48]. Its activity against multiple antibiotic-resistant pathogens from patients with cystic fibrosis is of particular interest [47].

SMAP28-NH₂ is thought to be the native form of the peptide [45,46] and synthesized SMAP28-NH₂ is antimicrobial [48]. Only recently, SMAP28-NH₂ has been isolated directly from ovine neutrophils (M. Ferguson, K. Brogden and B. Tack, unpublished observations).

The antimicrobial activity of cathelicidins is strongly dependent upon their compositions. In a recent study, the size and the amino acid composition of SMAP29 was systematically altered to create a family of 23 related peptides [48]. SMAP28, SMAP29, and a derivative of SMAP29 called ovispirin were all effective against Gram-negative bacteria (MIC range 0.1–10.0 mg/l), Gram-positive bacteria (MIC range 0.8 to >20.0 mg/l), and *Aspergillus fumigatus* (MIC 2.5 mg/l). Congeners of SMAP29 were not as active as the native peptide. Large congeners of ovispirin (>16 residues) were more active than smaller congeners (<16 residues) of ovispirin. However, substituting residues in the peptide or adding an N-terminus amine improved antimicrobial activity of the small congeners [48]. Ovispirins (OV-1, OV-2 and OV-3) were active against multiple antibiotic-resistant *P. aeruginosa*, *Burkholderia cepacia*, *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia* strains isolated from CF patients (MIC, 0.03–16 mg/l). Cathelicidin-derived peptides represent a novel class of antimicrobial agents and warrant further development as prophylactic or therapeutic agents for CF lung disease.

Table 3
Cathelicidins found in domesticated animals

Cathelicidin	Sequence	References
<i>Cattle</i>		
BMAP27	GRFKRFRKKKFKLKKLSPVIPLHLG	[124]
BMAP28	GGLRSLGRKILRAWKKYGPIIPIRIG	[124]
BMAP34	GLFRRLRDSIRRGQQKILEKARRIGERIKDIFRG	[34]
Bac5	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPPFRPPLGPFPGRR	[54,56]
Bac7	RRIRPRPRLRPRPRPLPFPRPGRPIPRPLPFPRPGRPIPRPLPFPRPGRPIPRPL	[34,44]
Indolicidin	ILPWKWPWWPWRG	[34,69,70]
Dodecapeptide	RLCRIVVIRVCR	[34,126,127]
<i>Sheep</i>		
OaBac5 α	RFRPXIRRPPIRPPFRPPFRPPVRPPIRPPFRPPFRPPIGPFPGRR	[54,128]
OaBac5 β	RFRPXILRPPIPPFXPPFXPPVRPPIRPPFRPPFRPPIGPFPGRR	[54,128]
OaBac6	RRLRPRHQHFPSERPWPKPLPLPRPGRPPWPKPLPLPRPGLRPWPKPL	[128]
OaBac7.5	RRLRPRRRLRPRRPRRPRSLPLPRPQPRRIPRILLPWRPPRPIPRPQPIPRWL	[44,128]
OaBac11	RRLRPRRRLRPRRPRRPRSLPLPRPKPRPIPRPLPLPRRPRKPIPRPLPLPRRPRRIPR- PLPLPRRPRPIPRPLPLPQPQPSPIPRPL	[128]
SMAP29	RGLRRLGRKIAHGKVKYGPVTLRIIRIAG	[11,43–46]
SMAP34	GLFGRLRDSLQRGGQKILEKAERIWCKIKDIFRG	[11,43–46]
Dodeca-peptide	RICRIIFLRVCR	[44]
<i>Goats</i>		
ChBac5	RFRPPIRRPPIRPPFNPPFRPPVRPPFRPPFRPPFRPPIGPFPGRR	[54]
<i>Pigs</i>		
PMAP23	RIIDLLWRVRRPQKPKFVTWVWR	[40,130,131]
PMAP36	GRFRRLRKKTRKRLKKIGKVLKWIPIVGSIPLGCG	[40,41,130]
PMAP37	GLLSRLRDFLSDRGRRLGEKIERIGQKIKDLSEFFQS	[41,131]
Cecropin P1	SWLSKTAKKLENSAKKRRISEGAIAIAIQGGPR	[38,39]
PR-39	RRRPRPPYLPRRPPFFPPRLPPRIPPGFPPRFPPRFPP	[38,132]
Protegrin-1	RGGRLCYCRRRFCVCVGR	[97]
Protegrin-2	RGGRLCYCRRRFCICV	[97]
Protegrin-3	RGGGLCYCRRRFCVCVGR	[97]
Protegrin-4	RGGRLCYCRGWICFCVGR	[41,134,138]
Protegrin-5	RGGRLCYCRPRFCVCVGR	[41,134,138]
Prophenin-1	AFPPNVPGPRFPPNFPGPRFPPNFPGPRFPPNFPGPRFPPNFPGPPFPPPIFPGPWFPFPPPP- FRPPPFGPPRF	[41,68]
Prophenin-2	AFPPNVPGPRFPPNFPGPRFPPNFPGPRFPPNFPGPRFPPNFPGPPFPPPIFPGPWFPFPPPP- FRPPPFGPPRF	[41,68]
<i>Horses</i>		
eCATH-1	KRFGRLAKSFLMRILLPRRKILLAS	[35,50]
eCATH-2	KRRHWFPLSFQEFLEQLRRFRDQLPFP	[35,50]
eCATH-3	KRFHVSGLIQRHQQMIRDKSEATR HGIRIITRPKLLLAS	[35,50]

SMAP29 induces extensive ultrastructural damage in bacterial cells [47,48]. SMAP29 induces bacterial membrane blebs within 1 min, kills *P. aeruginosa* within 1 h and causes a dose-dependent, reversible decrease in transepithelial resistance within 5 h [47]. In transmission electron microscopy, SMAP29 induces damage characterized by rough surfaces containing extracellular debris and outer membranous blebs, thickened cell walls and electron dense cytoplasmic material (Fig. 2). Interestingly, the ultrastructural changes induced by SMAP29 were different from that induced by other peptides like CAP18 [48]. In addition to severe membrane changes, SMAP29 rapidly penetrated the outer and inner membranes and entered into the bacterial cytoplasm as early as time 0 (Fig. 2).

BMAP34 is a peptide of 34 residues deduced from a bovine cathelicidin gene sequence [49]. BMAP34 is stored as a proform in the cytoplasmic granules of bovine neutrophils. CD spectroscopy and secondary structure analysis indicates that the peptide assumes an amphipathic alpha-helical conformation. The peptide exerts a broad-spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria and is not active against eukaryotic cells.

eCATH-1, eCATH-2 and eCATH-3 are cathelicidins identified in horse bone marrow cells by using RT-PCR to find the conserved cathelin-like propeptide [35]. Sequence analysis of amplified products suggested the presence of three different cathelin sequences [35] and the deduced C-terminus antimicrobial domains con-

Table 4
Defensins found in domesticated animals

Defensin	Sequence	References
<i>Cattle</i>		
BNBD-1	DFASCHTNGGICLPNRCPGHMIQIGICFRPRVKCCRSW	[77]
BNBD-2	VRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW	[77]
BNBD-3	pEGVRNHVTCRINRGFCVPIRCPGRTRQIGTCFGPRIKCCRSW	[77]
BNBD-4	pERVRNPQSCRWNMGVCIPFLCRVGMQRQIGTCFGPRVPCCRR	[77]
BNBD-5	pEVVRNPQSCRWNMGVCIPISCPGNMRQIGTCFGPRVPCCRR	[77]
BNBD-6	pEGVRNHVTCRIYGGFCVPIRCPGRTRQIGTCFGPRVKCCRRW	[77]
BNBD-7	pEGVRNFVTCRINRGFCVPIRCPGHRPQIGTCLGPRIKCCR	[77]
BNBD-8	VRNFVTCRINRGFCVPIRCPGHRRQIGTCLGPQIKCCR	[77]
BNBD-9	pEGVRNFVTCRINRGFCVPIRCPGHRRQIGTCLGPQIKCCR	[77]
BNBD-10	pEGVRSYLSCWGNRGICLLNRCPGRMRQIGTCLAPRVKCCR	[77]
BNBD-11	GPLSCRRNGGVCIPRCPGPMRQIGTCFGRPVKCCRSW	[77]
BNBD-12	GPLSCGRNGGVCIPRCPVPMRQIGTCFGRPVKCCRSW	[77]
BNBD-13	SGISGPLSCGRNGGVCIPRCPVPMRQIGTCFGRPVKCCRSW	[77]
TAP	NPVSCVRNKGICVPIRCPGSMKQIGTCVGRAVKCCRKK	[81,82,84]
LAP	QGVRSQNCRNKGICVPIRCPGSMRQIGTCLGAQVKCCRKK	[83]
EBD	GISNPLSCLNRGICVPIRCPGNLRQIGTCFTPSVKCCRWR	[80,123]
<i>Sheep</i>		
SBD-1	MRLHLLLLVLFVVLSAGSGFTQGVNRNLSCHRNGVCVPSRCPRHMRQIGTCRGGPPVKCCRKK (prepropeptide sequence)	[86]
SBD-2	MRLHLLLLVLFVVLSAGSGFTHGVTDSLSCRWKKGICVLTRCPGTMRQIGTCGGPPVKCCRLLK (prepropeptide sequence)	[86]
<i>Goats</i>		
GBD-1	GSGFTQGIRRRSCHRNGVCALTRCPNMRQIGTCFGPPVKCCRKK	[139]
GBD-2	GSGFTQGIINHRSCYRNKGVCAPARCPNMRQIGTCHGPPVKCCRKK	[139]
<i>Pigs</i>		
PBD-1	KNIGNSVSLRNKGVCMPGKCAPKMKQIGTCGMPQVKCCRKK	[89]
<i>Poultry</i>		
Gal 1 α	GRKSDCFRKNGFCAFLKCPYLTLISGKCSRFLHCKRIWG	[91,93]
CHP2		
Gal 1/	GRKSDCFRKSFGFAFLKCPSLTLISGKCSRFLYLCCKRIWG	[90,91,93]
CHP1		
Gal 2	LFC KGGSCHFGGCPSHLIKVGSCFGFRS CCKWPWNA	[90,91,93]
Gal 3	ATQCRIRGGFCRVGSCRFPHIAIGKCATFISCCGRAYEVDALNSVRTSPWLLAPGNNPH	[93]
THP 1	GKREKCLRNRNGFAFLKCPVTLVSGTCSRFGVCCCKTLLG	[90,93]
THP 2	LFCKRGTCFHGRCPSHLIKVGSCFGFRSCKWPSDA	[90,93]
GPV-1	TPIQCRIRGGFCRVGSCRFPHIAIAKCATFIPCCGSIWG	[93]

tained 26, 27, and 40 residues (Table 3). Western blot analysis with antibodies to synthetic eCATH peptides suggested that eCATH-2 and eCATH-3 are functional gene products, whereas eCATH-1 is not. These peptides are thought to be stored as unprocessed forms in equine neutrophils and when released during inflammation, are cleaved to yield eCATH-2 and eCATH-3 as well as two smaller forms of eCATH-3 called eCATH-3₅₋₄₀ and eCATH-3₇₋₄₀ [50]. When synthesized and tested for their antimicrobial activity, eCATH-1 was the most effective against Gram-positive and Gram-negative bacteria. eCATH-2 displayed up to 16-fold higher MICs than eCATH-1 and eCATH-3. eCATH-3₅₋₄₀ and eCATH-3₇₋₄₀ were ineffective [50].

2.2.2. Linear peptides, rich in proline

These peptides are 40–80 residues in size, linear without cysteine residues and may form extended coils. They have a high proportion of select residues, particularly proline, arginine, glycine or tryptophan. Bactenecins (Bac5, Bac7), PR-39, prophenin and indolicidin are found in this group (Table 1).

Bactenecins are arginine-rich antibacterial peptides from bovine [51–53], ovine [54] and caprine [54] neutrophil granules. In cattle, they are containing a high content of proline (greater than 45%) and arginine (greater than 23%) residues [52,53]. Bac5 (sometimes called PR-42) has 42 amino acid residues and a mass of 5000 Da. The peptide has a repeated motif of Arg-Pro-

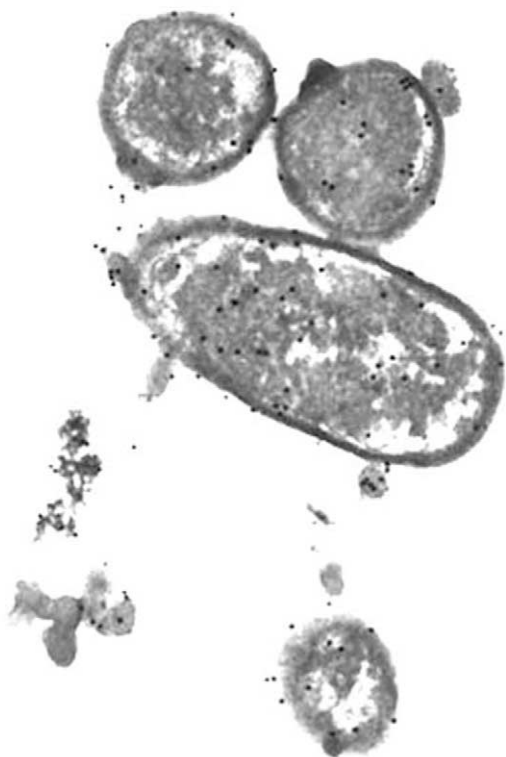


Fig. 2. *P. aeruginosa* incubated with SMAP29. Specific antibody and Protein A colloidal gold labelling demonstrate the rapid penetration of the peptide into the bacterial cell. Note the extensive outer membrane material that has been 'scrubbed' from the bacterial cell surface [48].

Pro triplets alternating with single apolar residues [52]. Bac7 (sometimes called PR-59) has 59 residues and a mass of 7000 Da. The peptide has three tandem repeats of a tetradecamer characterized by several Pro-Arg-Pro triplets spaced by single hydrophobic amino acids [52]. A Bac4 molecule has been found but is thought to be non-functional, and synthetic Bac4 has no antibacterial activity [55]. Bac5 and Bac7 are stored as proproteins of 20 and 16 kDa, respectively, in large cytoplasmic granules present in the neutrophils [56,57] and become activated by proteases in the azurophilic granules when these granules fuse with the probactenecin-containing granules [57]. Both Bac5 and Bac7 have potent antimicrobial activity *in vitro* [52,53].

In sheep, there is some heterogeneity in Bac5. Two proline-rich peptides, OaBac5 α and OaBac5 β , were isolated from elastase-treated extracts of sheep leukocytes [54]. OaBac5 β was 20–30% as abundant as OaBac5 α , 38 Da smaller and differed from it in 2 of 17 residues [54]. OaBac5 α and ChBac5 both exhibited potent, broad-spectrum antimicrobial activity.

PR-39, originally isolated from the upper part of the small intestine of the pig, contains high concentrations of proline (45–49%) and arginine (24–29%) [58,59]. Different forms occur, and PR-39 isolated from porcine

small intestine is slightly different in composition from that isolated from porcine neutrophils [59]. A variant form of PR-39, called β PR-39, differs by 3.6%, reflecting the occasional base pair substitutions scattered throughout the gene [60]. PR-39 is directly involved in the host response to infection, and pigs infected with *Salmonella choleraesuis* had serum concentrations of PR-39 increase threefold over prechallenge concentrations of 13.6 ± 1.0 ng/ml [61]. Synthetic and natural forms of the peptide are active for Gram-negative bacteria only and MICs range from 1–4 μ M [58,62]. However, PR-39 is active against drug-susceptible as well as multi-drug-resistant clinical isolates of *Mycobacterium tuberculosis* [63].

PR-39 does not induce membrane lysis nor kills bacteria by a non-pore forming mechanism (Table 5). Instead, it kills bacteria by a mechanism that stops protein and DNA synthesis after a lag period of about 8 min [58]. Growing bacteria are killed faster than non-growing bacteria [58]. A charged N-terminus is important for peptide activity and the functional antibacterial domain is in the first 26 amino acid residues of the N-terminus [59].

PR-39 also affects cell development, differentiation and metastatic transformation [64]. PR-39 inhibits the NADPH oxidase activity of neutrophils by blocking assembly of this enzyme [65]. Again, the presence of a charged N-terminus is important for these peptide activities in mammalian systems. For example, PR-39 is a calcium-dependent chemoattractant of neutrophils but not mononuclear cells, and the chemotactic domain is in the first 26 amino acid residues [66].

Prophenin is another antimicrobial peptide isolated from porcine leukocytes [60,67,68]. The name reflects the abundance of proline (57.1%) and phenylalanine (19.0%) residues. Two forms exist, prophenin-1 [67] and prophenin-2 [60,67], that differ in their compositions. Prophenin-1 is an 8683 Da peptide of 79 residues encoded on a cathelin-containing precursor [67]. It contains 42 (53.2%) proline and 15 (19.0%) phenylalanine residues. The N-terminal 60 residues consist of three perfect and three nearly perfect repeats of a decamer, FPPPNFPGPR. Prophenin-1 is substantially more active *in vitro* against *E. coli* than against *Listeria monocytogenes* [67].

Table 5
Mechanisms of antimicrobial activity of peptides from domesticated animals

Antimicrobial activity	Peptide	References
Ion channel formation	cecropins	[58,140]
	defensins	[141]
Inhibition of DNA and protein synthesis	PR-39	[58]
Effect on transport and energy metabolism in cytoplasmic membrane	Bac5, Bac7	[51]
Flocculation of intracellular contents	anionic peptides	[22]

Indolicidin is a tryptophan-rich, 13 amino acid, antimicrobial peptide isolated from the large cytoplasmic granules of bovine neutrophils [69]. The deduced form from bovine marrow cells is a 14 amino acid peptide [70]. The glycine found at the C-terminus end of the peptide is not found in the purified peptide [69], suggesting that the deduced sequence is very likely involved in post-translational peptide amidation. The peptide contains five tryptophans, three prolines, three basic residues, and no acidic residues [69,71]. The MIC is less than 10 mg/l for both Gram-negative and Gram-positive bacteria [69]. Indolicidin is highly membrane-active and is believed to disrupt cell membranes by binding to phospholipids [71].

2.2.3. Cysteine-stabilized peptides with a β -sheet structure

These peptides are 16–40 residues in size, contain cysteines, have two or more disulphide bonds and form a stabilized β -sheet structure. This group contains the α -, β -defensins and protegrins (Tables 1 and 4).

α -Defensins, often called classical defensins, have 29–35 residues and are found in the azurophil granules of neutrophils of humans and many smaller mammalian species such as guinea pigs, rabbits, rats and mice [72]. These peptides are stored in high concentrations in granules, specialized organelles of phagocytes (granulocytes and some macrophages) and Paneth cells [72]. They are active at 1–100 mg/l against Gram-positive bacteria, Gram-negative bacteria, mycobacteria, fungi and enveloped viruses [72–75].

β -Defensins have 38–42 residues and are arginine-rich with broad antimicrobial, antiviral and cytotoxic activity [76]. In cattle, thirteen β -defensins are found in the granule-rich cytoplasmic fraction of purified blood neutrophils and are called bovine neutrophil beta defensins or BNBD [77]. These 13 homologous peptides are highly cationic and share conserved consensus sequence with epithelial antimicrobial peptides [77]. All were active against *S. aureus* and *E. coli* [77].

BNBD-4, BNBD-12 and BNBD-13 have been studied in detail [78,79]. Early work with BNBD-12 established that disulphide motifs of BNBD differed from that of classical defensins, showing that β - and α -defensins have differently folded chains, though they share several functional properties [79]. The 41 residue of BNBD-4 was found to originate from a 63 residue prepropeptide, whereas the 38 and 42 residues BNBD-12 and BNBD-13 originated from a common 60 residue prepropeptide [78]. Two-exon genes that are closely related to bovine epithelial defensin genes encode the peptides. BNBD-4 and BNBD-12/13 mRNAs are abundant in bone marrow but are expressed differentially in certain non-myeloid tissues [78]. BNBD-12 is in dense granules and is the predominant organelle form in myeloid cells. In bovine alveolar macrophages, BNBD-4, BNBD-5,

TAP, and EBD are constitutively expressed [80]. Exposure to LPS did not induce BNBD expression in bovine alveolar macrophages but did induce expression of tumour necrosis factor alpha.

Two epithelial β -defensins, tracheal antimicrobial peptide (TAP), and lingual antimicrobial peptide (LAP) are found in columnar cells of the pseudostratified epithelium throughout the conducting airway and tongue [81–84]. TAP is expressed in nasal, tracheal and bronchiolar tissues [84]. However, mRNA encoding TAP is more abundant in the respiratory mucosa than in whole lung tissue [82] and is not expressed in alveolar sections or submucosal glands [84]. TAP is not expressed prenatally in foetal calves [84]. LAP was isolated from bovine tongue and has a broad-spectrum of antibacterial and antifungal activities [83].

TAP gene expression dramatically increases in primary cultures of bovine airway epithelial cells incubated with LPS [84,85]. At 10 ng/ml, there was a 5- to 10-fold increase in TAP expression [85]. Similarly, LAP mRNA was markedly increased in the epithelium surrounding naturally occurring tongue lesions. This increase coincided with the cellular hallmarks of acute and chronic inflammation in the underlying lamina propria, supporting its role as an integral component of the inflammatory response.

In sheep, SBD-1 and SBD-2 are expressed throughout the gastrointestinal tract [86]. SBD-1 mRNA is the most prevalent from the tongue to colon with the exception of the distal ileum, where SBD-2 mRNA predominates. SBD expression also varies significantly between animals and is developmentally regulated both pre- and post-natally. High resolution chromosomal fluorescence in situ hybridization (FISH) and R-banding localized the β -defensin cluster to chromosome band 24q13 [87].

In goats, two β -defensin precursors, preproGBD-1 and preproGBD-2, are also found [88]. They are identical in 96.8% of their bases and 88.2% (60 of 68) of their amino acids. However, preproGBD-1 is expressed principally in the tongue and respiratory tract, whereas preproGBD-2 is expressed throughout the intestine.

In pigs, PBD-1 is expressed in the cornified tip of the filiform papillae of the dorsal tongue and in the superficial squamous cell layers of the buccal mucosa [89]. The cDNA sequence of PBD-1 encodes a 64 amino acid prepro-peptide, which contains the 42 residue natural form [89]. mRNA was detected throughout the respiratory and digestive tracts and also in thymus, spleen, lymph node, brain, liver, kidney, urinary bladder, testis, skin, heart, muscle, bone marrow, peripheral blood neutrophils, alveolar macrophages and umbilical cord. The peptide is antimicrobial at 40 mg/l, and activity is synergistic with other neutrophil-derived antimicrobial peptides, such as PG-3 and PR-39 [89].

In poultry, four chicken β -defensins (Gal 1/CHP1, Gal 1 α /CHP2, Gal 2, and Gal 3) and four turkey β -defensins (THP 1, THP 2, THP 3 and GPV-1) have been reported [90–93]. The peptides contain 36–39 amino acid residues and are relatively cationic due to their numerous lysine and arginine residues. Three intramolecular cystine disulphide bonds are present [91]. These peptides were >90% effective at 2–16 mg/l concentrations against *E. coli* and *L. monocytogenes* [91,92]. In normal chickens, the expression of Gal-3 is especially prominent in the tongue, bursa of Fabricius and trachea [93] but also occurs in the skin, oesophagus, air sacs, large intestine and kidney. Tracheal expression of Gal 3 increased significantly after experimental infection of chickens with *Haemophilus paragallinarum*, whereas its expression in the tongue, oesophagus, and bursa of Fabricius was unaffected. The precursor of Gal 3 contains a long C-terminal extension not present in the prepropeptide. By comparing the cDNA sequences of Gal 3 and GPV-1, it appears that a 2-nucleotide insertion into the Gal 3 gene has induced a frameshift that reads through the original stop codon and allows the chicken propeptide to lengthen.

Another defensin-like molecule was recently isolated from the granules of neutrophils and monocytes of rhesus macaques [94]. This peptide is an 18-residue macrocyclic, trisulphide antibiotic peptide termed rhesus theta defensin-1 (RTD-1). The peptide backbone is cyclized through peptide bonds with an extended beta-hairpin structure with turns at one or both ends [95]. In contrast to numerous other antimicrobial peptides, RTD-1 does not display any amphiphilic character, even though surface models of RTD-1 exhibit a certain clustering of positive charges [95]. It is microbicidal for bacteria and fungi at low micromolar concentrations. Antibacterial activity of the cyclic peptide was threefold greater than that of an open-chain analog, and the cyclic conformation was required for antimicrobial activity in the presence of 150 mM sodium chloride. Biosynthesis of RTD-1 involves the head- to-tail ligation of two α -defensin-related nonapeptides, requiring the formation of two new peptide bonds.

Animal protegrins consist of five cysteine-rich naturally occurring cationic antimicrobial peptides [96]. In porcine leukocytes, protegrins contain 16 residues with four cysteines stabilized by two intramolecular disulphide bonds [97]. Although the primary amino acid sequences of the protegrins show minimal homology to the β -defensins, the first 3-cysteine residues are spaced identically to those of α -defensins [97]. Two antiparallel β -sheets are linked by a β -hairpin turn [96]. In artificial membranes, they form dimeric structures, which aggregate forming a larger pore [96]. Protegrins are stored as inactive proforms in neutrophil granules and are activated extracellularly by neutrophil elastase [98]. They

have potent activity against Gram-negative bacteria, Gram-positive bacteria and fungi [97].

2.3. Peptides as fragments of larger proteins

One final area worth mentioning includes the isolation of antimicrobial domains often residing in larger antimicrobial proteins. These domains are thought to be natural enzymatic digestion products of larger molecules, perhaps consumed and degraded in the intestinal tract. These include lysosome residues 98 through 112 [99], a lactoferrin fragment called lactoferricin [100–104] and hen ovotransferrin residues located within the 109–200 sequence of the N-terminus [105]. These studies suggest that ingested bovine lactoferrin is partially degraded by gastric contents to antimicrobial fragments containing the lactoferricin region [103]. These peptides then exhibit broad-spectrum antimicrobial activity in the gastrointestinal tract [100,101,104].

3. Therapeutic potential

The highly active nature of antimicrobial peptides *in vitro* suggests these peptides might be effective in treating bacterial infections *in vivo*. To test this, a model of acute pneumonia in lambs was established using the ovine respiratory pathogen *M. haemolytica*. In one study, a single bronchial instillation of 0.5 mg SMAP29 reduced the concentration of bacteria in both the bronchoalveolar lavage fluid and consolidated pulmonary tissues [43](Fig. 3). In another study, a single bronchial instillation of 0.5 mg anionic peptide also reduced pulmonary inflammation and the concentration of *M. haemolytica* in infected lung tissue [106]. The *in vitro* and *in vivo* effectiveness of SMAP29 and anionic peptides suggests that they may have applications in the treatment or prevention of pulmonary infections. However, further studies are needed to confirm these findings and also to determine the optimal doses and intervals of antimicrobial peptide therapy.

4. Future areas of research

The field of antimicrobial peptides in animals is expanding and substantial advances are expected. First, new genes encoding antimicrobial peptides will likely be found in sequenced animal genomes. For example, four β -defensins were discovered in human tissues, urine, and secretions [107–112]. Recently, Schutte et al., using a genomics approach, identified 28 new human β -defensin and 43 new mouse β -defensin genes in five syntenic chromosomal regions. Within each syntenic cluster, the gene sequences and organization were similar, suggesting each cluster pair arose from a common ancestor and

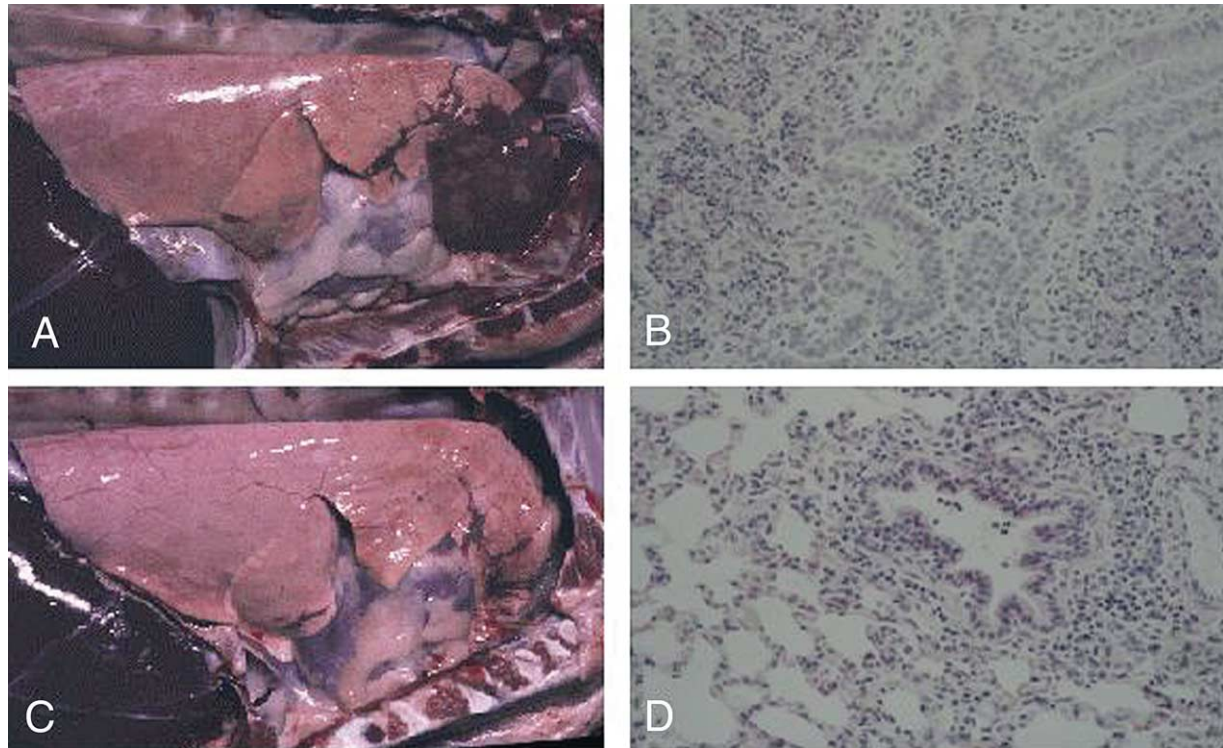


Fig. 3. Lungs of lambs infected with *M. haemolytica* [43]. The anterior part of the right cranial lobe (A) of infected lambs consisted of areas of consolidation with necrosis and haemorrhage. Lumens contained large numbers of neutrophils, cell debris and proteinaceous exudate (B). The lesion is significantly reduced 24 h after a single treatment with SMAP29 (C). The bronchiolar walls contained moderate numbers of lymphocytes, but there was no acute inflammatory response (D).

was retained because of conserved functions [113]. The discovery of these novel β -defensin genes exemplifies how sequencing of animal genomes may also identify new antimicrobial gene products.

Second, conventional animals or transgenic animals will be likely be used as a source of antimicrobial peptides for use in the food and health industry. Bovine lactoferrin, a byproduct from cheese whey or skim milk, is commercially available as a health food in Japan [114] and has been used in a wide variety of products since it was first added to infant formula in 1986 [115]. Similarly, recombinant human lactoferrin (rhLF), was found in transgenic cow's milk at gram per litre concentrations [116]. Natural hLF from human milk and rhLF had identical iron-binding and iron-release properties and were equally effective in three different in vivo infection models employing immunocompetent and leukocytopenic mice [116]. Lactoferrins have little toxicity and are very safe, especially when administered orally. The oral toxicity of purified lactoferrin is extremely low and 2000 mg/kg/day did not cause any adverse effects in rats of both sexes [115,117]. Similarly, 434 mg/kg lactoferrin was not toxic for mice [118]. Based

on these results, lactoferrin was considered to be a highly safe food additive in supplemented infant formula, milk, skim milk, yoghurt, chewing gum, nutritional supplements, skin care cosmetics, therapeutic diet for relief of inflammation in dogs and cats and in aquaculture feed [115].

Third, antimicrobial peptides have immunoenhancing activities. For example, human neutrophil peptides (HNP) can induce adaptive immune responses to foreign antigens. Lillard et al. showed that a mixture of native HNPs (containing HNP-1, HNP-2 and HNP-3) induced adaptive host immunity to ovalbumin in mice [119]. Intranasal delivery of HNPs plus ovalbumin enhanced ovalbumin-specific IgG, but not IgA, antibody responses. Ovalbumin specific T cells showed marked increases in ovalbumin-specific proliferative responses and produced greater amounts of CD4⁺ Th1 and Th2 cytokines (interferon- γ , IL-5, IL-6 and IL-10). Similar work by Brogden et al. showed that HNPs and HBDs could also induce adaptive immune responses to ovalbumin [120]. Different antibody isotype and cytokine profiles, unique to the individual HNP or HBD used, were seen. Such immunoenhancing properties could be

used to augment or direct favourable immune responses to co-administered antigens in vaccines for prophylactic prevention of disease [120].

5. Summary

In this review, we presented an updated list of antimicrobial peptides found in domesticated animals including their sites of production, antimicrobial activity and their potential use for therapeutic treatment of infectious disease. These antimicrobial peptides are conserved in their structure, function and mechanisms of action, and thus it might be possible to use them to prevent or treat microbial infections. First, these peptides are generally broad-spectrum for Gram-positive bacteria, Gram-negative bacteria and fungi. Synthetic antimicrobial peptides or their congeners might be used directly as antimicrobial agents to treat infections. Preliminary studies presented here suggest that this may be possible. Second, as we begin to identify the mechanisms to initiate synthesis and release of antimicrobial peptides, it may also be possible to find ways to trigger their production in animals at times when they are most susceptible to microbial infection. Such prophylactic treatment may help animals through stressful periods (e.g. exposure to inclement weather, poor ventilation with high levels of moisture and barnyard gases, handling and transport, castration and docking, weaning and change in feed, high loads of parasites and mixing of animals from different sources). Third, it may be possible to use virus-mediated gene transfer to express antimicrobial peptides in pulmonary epithelia before or during infectious disease outbreaks. This would be particularly applicable in situations involving respiratory infections. Finally, it is possible that antimicrobial peptides could be used as mucosal adjuvants to augment or direct favourable immune responses to co-administered antigens in vaccines for prophylactic prevention of disease [120].

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