

Isolation and engineering of antimicrobial peptides targeting pathogenic *Escherichia coli* and *Salmonella* of poultry

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Supplementary information

Table S1. Summary of NGS analysis of a 16mer pIII-display peptide library

% of singletons ¹	90.573
% of 2 copies	8.575
% of 3-5 copies	0.848
% of 6-24 copies	0.004
% of >25 copies	ND ²

¹2.6 million sequences were analysed that contained the two flanking regions in frame with each other either side of a 16 amino acid insert. ²None detected.

Table S2. Enrichment levels of selected peptides from NGS analysis.

Peptide	Enrichment score ¹
EQIWLHLFRLINQSLC	25
WRGTALKFICYPVLLL	25
RFISEAQDQICIPLRA	25
PVWFVQSLRPKNTCVE	25
LWNWPGVLTCLVCLIP	24
VLCSLLQSVPLFERVW	23
GTSFLVCGLSIFVIFK	21
WSSGFRWLTCLLTWH	21
LLGWYTPLECFRKLK	21
GPTVMSMWKPFPSLA	18
LTTVVCAIWHVLTTPSH	17
IGYWLSDCVLPWLLQT	17
WGSKLLQVFSTICNMV	16
YFLHHWVPLWCTLHM	16
GPTVMSMWKPFSLFSLA	15
PVGACVYILSSFAPIA	15
AGDYCLFLCQLSRLA	15
RQTVLSWILCYSDRWW	15
GTSFWSVVOVFCNLK	15
LGTHFSELFQHWPLR	15

SHVVNLNWLRLWLYCSL	15
WFQRIDVFFQTPFLLF	15
STYHWHNFIYFVQCIL	15
GPTVMSMWKPFSLLVFA	14
LLGVGGILRWSVFWVSV	14
WGSKVNFPRIWCLLH	14
APGWWRIAPFLQGPFC	25
GMFGGLCNPWIFAHF	25
EVSWLWGIWPSWQVE	25
VWEIGPRWWTQLTCPS	25
FHWPFPRFAWLDHGYC	25
WRERLSAPGCFLAYLL	24
VVSSWPWLLAHVSCWI	24
SFGVRQLLCMMTGFWH	24
PNQIYRSRQWVECLLF	24
SLKLVPSFPFGNSICQ	23
LGKLRTFICQLDYILV	23
FADFMIQMACRANLFV	23
LLHRMVCLSAQAADWL	23
TYWADWWRLGGREPC	23
WLDLAWCMLRANAFSF	23
GWGKWGLRQLRDFAVC	22
AGSLFRSVIDLVCVWG	22
PFWGGEALKWLGFRAC	22
VREVVCWAVWAVQGV	22
RVPDLFDVLCHHKGSQ	22
FGQSLRTWVLSLWCYD	22
FMGWVFNWLSCGYHGL	22
WDEAVVGYLRQFWCWA	22
QILGKVWKSIDWFLCD	21
SLNSVLSLLDNEYHPN	21
SVQDFVCRYMSLFFST	21
FWDDWLFWARAGLCAL	21
SVPLRWLCSDNFYQKN	21
STEARVPLMRHVCSL	21
AFVPPRPAFLHKLFL	20
VSGLRIWLRDTACAVL	20

¹Selected peptides were ranked by the number of z-values >2 calculated for relative enrichment between 5 replicates of the target bacteria and 5 replicates of the control bacteria (score out of 25). This was carried out separately for panning against *E. coli* O2 (shaded) and *S. Typhimurium*. Sequences in bold were shown to have AMP activity in this study.

Table S3. MIC data for monomers.

Peptide	Peptide concentration (mg/L)	Bacteria species/serotype ¹			
		<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. T.</i>	<i>S. E.</i>
WGS	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
WRE	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
VVS	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
GWG	4				
	8	3			
	16	5	9		
	32	1		3	
	64			6	2
	128				7
	>128				
AFV	4				
	8				
	16		2		
	32		2		
	64		5		
	128				
	>128	9		9	9
C-BF	0.0625				
	0.125				
	0.25	1	2		
	0.5	3	4	9	6
	1	5	3		3
	2				

¹Three MIC assays, independent of each other, were carried out in triplicate against each bacteria. The lowest concentration of peptide in each of the nine repeats which caused >50% inhibition of growth is shown, the modal value (in bold) was used as the MIC. *S.T.*: *S. Typhimurium*; *S.E.*: *S. Enteritidis*.

Table S4. MBC data for monomers.

Peptide	Peptide concentration (mg/L)	Bacteria species/serotype ¹			
		<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. T.</i>	<i>S. E.</i>
WGS	4				
	8				
	16				
	32				
	64				
	128				4
	>128	6	6	6	2
WRE	4				
	8				
	16			2	
	32			4	
	64				
	128				
	>128	6	6		6
VVS	4		1		
	8	6	2		
	16		3	2	
	32			4	
	64				
	128				
	>128				6
AFV	4				
	8				
	16				
	32				
	64				
	128				
	>128	6	6	6	6
GWG	4	4²	2		
	8				
	16	1	4		
	32	1		2	
	64			4	
	128				
	>128				6
C-BF	4			6²	
	8		3		
	16	2			
	32	4	3		
	64				
	128				
	>128				6

¹Two MBC assays, independent of each other, were carried out in triplicate against each bacteria. The lowest concentration of peptide in each of the six repeats which caused a drop of viable bacteria of >3-log₁₀ is shown, the mode of the six values (in bold) is reported as the MBC value. *S.T.*: *S. Typhimurium*; *S.E.*: *S. Enteritidis*.²The MBC was 4 mg/L or lower.

Table S5. The effects of AMP monomers on mixed cultures of *E. coli* O78 and *S. Enteritidis*

Peptide	Peptide concentration (mg/L)	Assay outcome ¹	
		<i>E. coli</i> O78	<i>S. Enteritidis</i>
GWG -experiment 1	2	NE	NE
	8	K	NE
	32	K	NE
GWG -experiment 2	4	I	NE
	16	K	NE
	64	K	NE
GWG -experiment 3	2	NE	NE
	8	K	NE
	64	K	NE
C-BF -experiment 1	1	K	NE
	4	K	K
	16	K	K
C-BF -experiment 2	0.25	K	NE
	1	K	NE
	4	K	K
C-BF -experiment 3	0.63	K	NE
	5	K	K
	40	K	K

¹K, killed - bacteria were detected at $< 5 \times 10^5$ /ml; I, inhibition of growth - bacteria were detected at between 5×10^5 /ml and 1×10^8 /ml; NE, no effect - bacteria were detected at $> 1 \times 10^8$.

Table S6. The effects of AMP monomers on mixed cultures of *E. coli* O2 and *S. Typhimurium*

Peptide	Peptide concentration (mg/L)	Assay outcome ¹	
		<i>E. coli</i> O2	<i>S. Typhimurium</i>
GWG -experiment 1	16	K	NE
	64	K	I
	256	K	K
GWG -experiment 2	16	I	NE
	64	K	K
	256	K	K
GWG -experiment 3	4	NE	NE
	16	K	NE
	64	K	I
C-BF -experiment 1	1	K	K
	4	K	K
	16	K	K
C-BF -experiment 2	0.5	K	NE
	2	K	K
	8	K	K
C-BF -experiment 3	0.25	K	NE
	1	K	I
	4	K	K

¹K, killed - bacteria were detected at $< 5 \times 10^5$ /ml; I, inhibition of growth - bacteria were detected at between 5×10^5 /ml and 1×10^8 /ml; NE, no effect - bacteria were detected at $> 1 \times 10^8$.

Table S7. MIC data for dimers.

Peptide	Peptide concentration (mg/L)	Bacteria species/serotype ¹			
		<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. T.</i>	<i>S. E.</i>
(WRE)-(WRE)	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
(WRE)-(C-BF)	4				
	8				
	16	4	8		
	32	1	1		
	64	3		6	1
	128			3	2
	>128				6
(VVS)-(VVS)	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
(VVS)-(C-BF)	4				
	8				
	16				
	32				
	64		6		
	128		3		
	>128	9		9	9
(GWG)-(GWG)	4				
	8				
	16				
	32				
	64				
	128				
	>128	9	9	9	9
(GWG)-(C-BF)	4				
	8				
	16		3		
	32	2	6		
	64	4		6	3
	128	3		3	6
	>128				
(C-BF)-(C-BF)	4				
	8	4		1	
	16	2	3	5	2
	32		5	3	4
	64	3	1		
	128				3
	>128				

¹Three MIC assays, independent of each other, were carried out in triplicate with peptide in the concentration range shown against each bacteria. The lowest concentration of peptide in each of the

nine repeats which caused >50% inhibition of growth is shown, the modal value (in bold) was used as the MIC. *S.T.*: *S. Typhimurium*; *S.E.*: *S. Enteritidis*.

Table S8. MBC data for dimers.

Peptide	Peptide concentration (mg/L)	Bacteria species/serotype ¹			
		<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. T.</i>	<i>S. E.</i>
(WRE)-(WRE)	4				
	8				
	16				
	32				
	64			1	
	128				
	>128	6	6	5	6
(WRE)-(C-BF)	4	6²	6²	3	
	8			3	
	16				1
	32				2
	64				3
	128				
	>128				
(VVS)-(VSS)	4				
	8				
	16				
	32				
	64				
	128				
	>128	6	6	6	6
(VVS)-(C-BF)	4		4²		
	8		2	4	
	16			2	3
	32				3
	64				
	128				
	>128	6			
(GWG)-(GWG)	4				
	8			1	
	16			4	
	32	1		1	
	64	5			
	128				
	>128		6		6
(GWG)-(C-BF)	4	6²	2	3²	
	8		4	1	
	16			2	
	32				
	64				
	128				
	>128				6

¹Two MBC assays, independent of each other, were carried out in triplicate against each bacteria. The lowest concentration of peptide in each of the six repeats which caused a drop of viable bacteria of >3-log₁₀ is shown, the mode of the six values (in bold) is reported as the MBC value. *S.T.*: *S. Typhimurium*; *S.E.*: *S. Enteritidis*. ²The MBC was 4 mg/L or lower.

Table S9. The effects of AMP dimer (GWG)-(C-BF) on mixed cultures

Peptide	Peptide concentration (mg/L)	Assay outcome ¹	
		<i>E. coli</i> O2	<i>S. Typhimurium</i>
Experiment 1	32	K	NE
	128	K	K
	512	K	K
Experiment 2	32	K	NE
	128	K	K
	512	K	K
Experiment 3	8	I	NE*
	32	K	NE*
	128	K	K

¹K, killed - bacteria were detected at $< 5 \times 10^5$ /ml; I, inhibition of growth - bacteria were detected at between 5×10^5 /ml and 1×10^8 /ml; NE, no effect - bacteria were detected at $> 1 \times 10^8$. Viable counts were carried out in duplicate with the exception of * where only a single repeat was analysed.

Table S10. AMP prediction tool CAMP-R4 was applied to peptides identified by NGPD

Peptide	CAMP-R4 vs natural ¹	CAMP-R4 vs synthetic ¹
EQIWLHLFRLINQSLC	0.34	0.38
WRGTALKFICYPVLLL	0.82	0.65
RFISEAQDQICIPRA	0.17	0.38
PVWFVQSLRPKNCTVE	0.33	0.09
LWNWPGVLTCLVCLIP	0.76	0.6
VLCSLLQSVPFLFERVW	0.48	0.21
GTSFLVCGLSIFVIFK	0.86	0.35
WSSGFRWLTCLLTWH	0.65	0.44
LLGWYTPLECFRKL	0.53	0.55
GPTVMSMWKPFPSLA	0.44	0.14
LTTVVCAIWHVLTPSH	0.64	0.44
IGYWLSDCVLPWLLQT	0.42	0.29
WGSKLLQVFSTICNMV	0.7	0.27
YFLHHWVLPWLCTLHM	0.35	0.18
GPTVMSMWKPFSLFSLA	0.51	0.16
PVGACVYILSSFAPIA	0.62	0.37
AGDYCLFLCQLSRLA	0.44	0.3
RQTVLSWILCYSDRWW	0.39	0.34
GTSFWSVVOVFCNLK	0.85	0.49
LGTHFSELCQHWPLR	0.2	0.17
SHVVLNWLERWLYCSL	0.38	0.25
WFQRIDVFFQTPFLF	0.41	0.3
STYHWHNFIYFVQCIL	0.29	0.14
GPTVMSMWKPFSLLVFA	0.58	0.18
LLGVGGILRWSVFSWV	0.74	0.68
WGSKVNFPRIWCLLH	0.89	0.65
APGWWRIAPFLQGPF	0.58	0.42

GMFGGLCNPWIWFAHP	0.49	0.33
EVSWLGWGIWPSWQVE	0.35	0.43
VWEIGPRWWTQLTCPS	0.37	0.22
FHWPFPRFAWLDHGYC	0.32	0.4
WRERLSAPGCFLAYLL	0.68	0.52
VVSSWPWLLAHVSCWI	0.54	0.48
SFGVRQLLCMMTGFWH	0.41	0.11
PNQIYRSRQWVECLLF	0.27	0.13
SLKLVPSFPFGNSICQ	0.62	0.18
LGKLRTFICQLDYILV	0.75	0.55
FADFMIQMACRANLFV	0.31	0.19
LLHRMVCLSAQAADWL	0.35	0.17
TYWADWWRLGGREPC	0.37	0.48
WDLAWCMLRANAFSF	0.4	0.44
GWGKWGLRQLRDFAVC	0.83	0.89
AGSLFRSVIDLVCVWG	0.64	0.57
PFWGGEALKWLGFRAC	0.66	0.8
VREVVCWAVWAVQGV	0.48	0.49
RVPDLFDVLCHHKGSQ	0.04	0.17
FGQSLRTWVLSLWCYD	0.48	0.19
FMGWVFNWLSCGYHGL	0.46	0.37
WDEAVVGYLRQFWCWA	0.39	0.5
QILGKVWKSIDWFLCD	0.59	0.65
SLNSVLSLLDNEYHPN	0.3	0.24
SVQDFVCRYMSLFFST	0.17	0.06
FWDDWLFWARAGLCAL	0.45	0.54
SVPLRWLCSDNFYQKN	0.37	0.15
STEARVPLMRHVCSL	0.26	0.14
AFVPPRPAFLHKLFLCL	0.83	0.67
VSGLRIWLRDTACAVL	0.83	0.78

¹sequences were compared to databases of either synthetic or natural AMPs, using Random Forest analysis. Shaded boxes indicate a probability value >0.5 indicating a predicted AMP. Sequences in bold were shown to have AMP activity in this study.

Table S11. Barcodes for NGS samples analysed to identify enriched peptides against target bacteria. Raw NGS data for the 20 barcoded samples are openly available at <https://www.ncbi.nlm.nih.gov/sra/SRX34872468%5Baccn%5D> with scripts and barcodes available at https://github.com/UoN-ADAS-KGough/NGS_Scripts01

Panning sample	Barcode ID
Panning against <i>E. coli</i> O2 – <i>E. coli</i> O2 binding - replicate 1	TTCTCATTGAAC
Panning against <i>E. coli</i> O2 – <i>E. coli</i> O2 binding - replicate 2	TCGCATCGTTC
Panning against <i>E. coli</i> O2 – <i>E. coli</i> O2 binding - replicate 3	TAAGCCATTGTC
Panning against <i>E. coli</i> O2 – <i>E. coli</i> O2 binding - replicate 4	AAGGAATCGTC
Panning against <i>E. coli</i> O2 – <i>E. coli</i> O2 binding - replicate 5	CTTGAGAATGTC
Panning against <i>S. Typhimurium</i> – <i>Salmonella</i> binding - replicate 1	TGGAGGACGGAC
Panning against <i>S. Typhimurium</i> – <i>Salmonella</i> binding - replicate 2	TAACAATCGGC
Panning against <i>S. Typhimurium</i> – <i>Salmonella</i> binding - replicate 3	CTGACATAATC
Panning against <i>S. Typhimurium</i> – <i>Salmonella</i> binding - replicate 4	TTCCACTTCGC
Panning against <i>S. Typhimurium</i> – <i>Salmonella</i> binding - replicate 5	CTTGACACCGC
Panning against <i>E. coli</i> O2 – <i>L. acidophilus</i> binding - replicate 1	TTGGAGGCCAGC
Panning against <i>E. coli</i> O2 – <i>L. acidophilus</i> binding - replicate 2	TGGAGCTTCCTC
Panning against <i>E. coli</i> O2 – <i>L. acidophilus</i> binding - replicate 3	TCAGTCCGAAC
Panning against <i>E. coli</i> O2 – <i>L. acidophilus</i> binding - replicate 4	TAAGGCAACCAC
Panning against <i>E. coli</i> O2 – <i>L. acidophilus</i> binding - replicate 5	TTCTAAGAGAC
Panning against <i>S. Typhimurium</i> – <i>L. acidophilus</i> binding - replicate 1	TCCTAACATAAC
Panning against <i>S. Typhimurium</i> – <i>L. acidophilus</i> binding - replicate 2	CGGACAATGGC
Panning against <i>S. Typhimurium</i> – <i>L. acidophilus</i> binding - replicate 3	TTGAGCCTATTC
Panning against <i>S. Typhimurium</i> – <i>L. acidophilus</i> binding - replicate 4	CCGCATGGAAC
Panning against <i>S. Typhimurium</i> – <i>L. acidophilus</i> binding - replicate 5	CTGGCAATCCTC

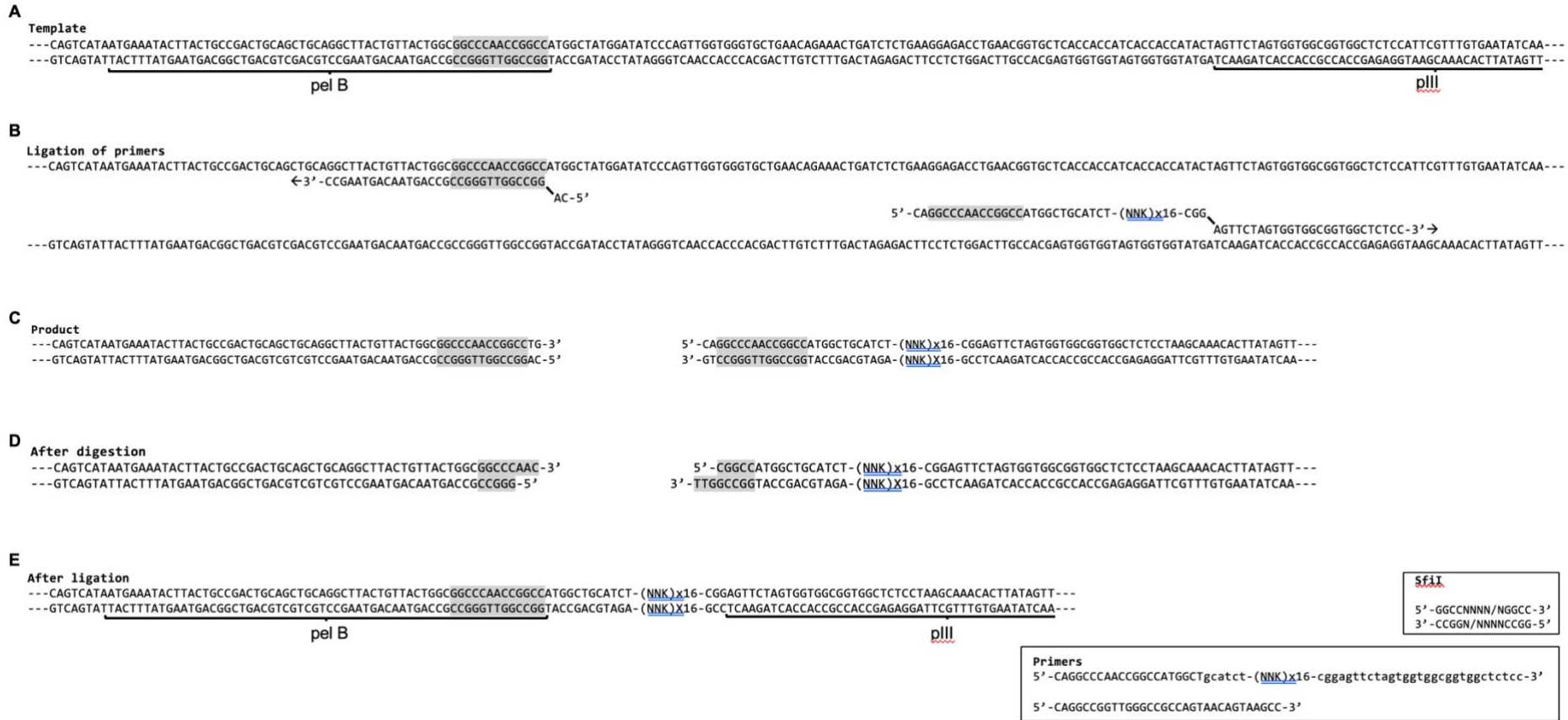


Figure S1. Phage display peptide library construction. The pSD3 phagemid vector was used as template (A) and the primers shown used for inverse PCR to remove the section between the pelB signal sequence and the truncated pIII gene (B), the Sfi cloning site is shaded. The inverse PCR primers either amplified over an existing SfiI site or introduced a new site (C). After SfiI digestion (D) the vector was ligated (E) to produce a gene coding for: MAAS-16mer-R-pIII for phage display.

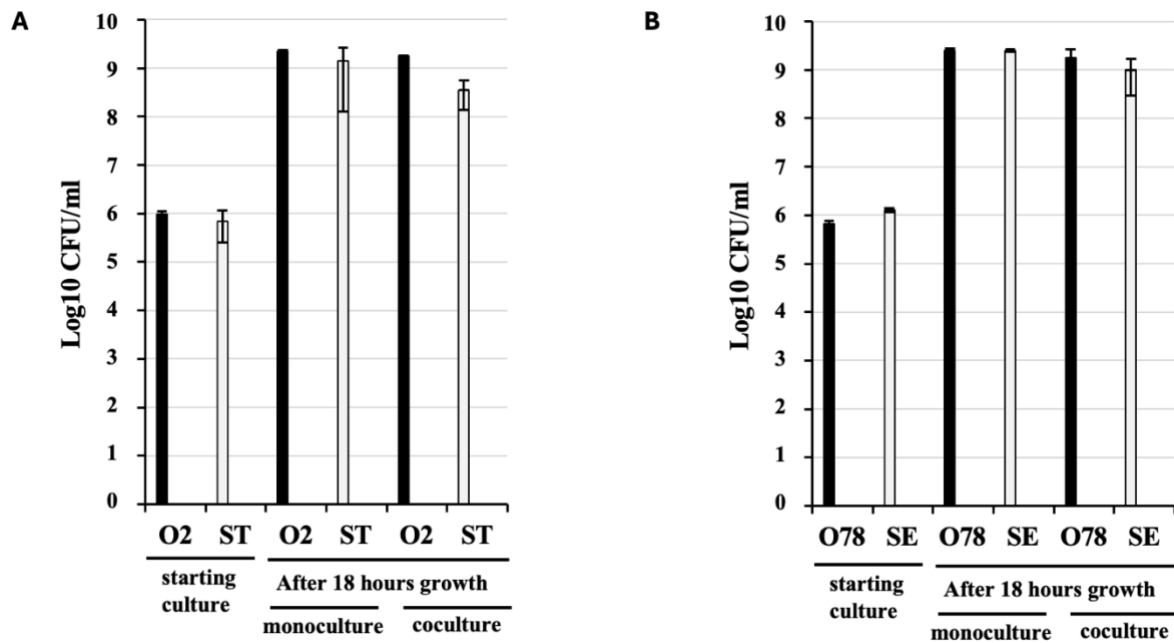
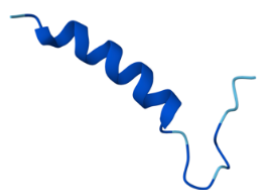
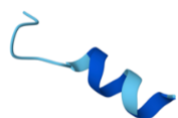


Figure S2. Effect of co-culture on bacterial titres. Cultures of either A) *E. coli* O78 and *S. Enteritidis* (SE) or B) *E. coli* O2 and *S. Typhimurium* (ST), were investigated for inhibitory effects due to co-culturing. After overnight incubation in MHB, from an initial inoculum of $\sim 10^6$ CFU/mL, a t-test was used to determine whether the viable counts of each bacterial isolate were significantly different when grown in a mixed culture compared to in a monoculture. No significant differences were found. Selective plating for *E. coli* O78 was on MHA supplemented with carbenicillin while selective plating for *Salmonella* was on MHA supplement with nalidixic acid. Viable counts of *E. coli* O2 were obtained by subtracting *Salmonella* counts (taken from nalidixic acid plates) from total viable counts on MHA. Viable counts were taken from duplicate samples.

C-BF



WGS



WRE



VVS



GWG



AFV



linker

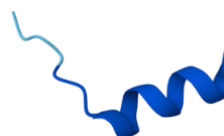


Figure S3. AlphaFold 3 structure predictions for AMPs. Secondary structure predictions are shown for each AMP. The linker used to construct dimers is also shown. Confidence in predicted structure is shown by the colour of the structure: Predicted Local Distance Difference Test values (pLDDT) - very high: >90, dark blue; confident: 70-90, light blue; low: 50-70, yellow.

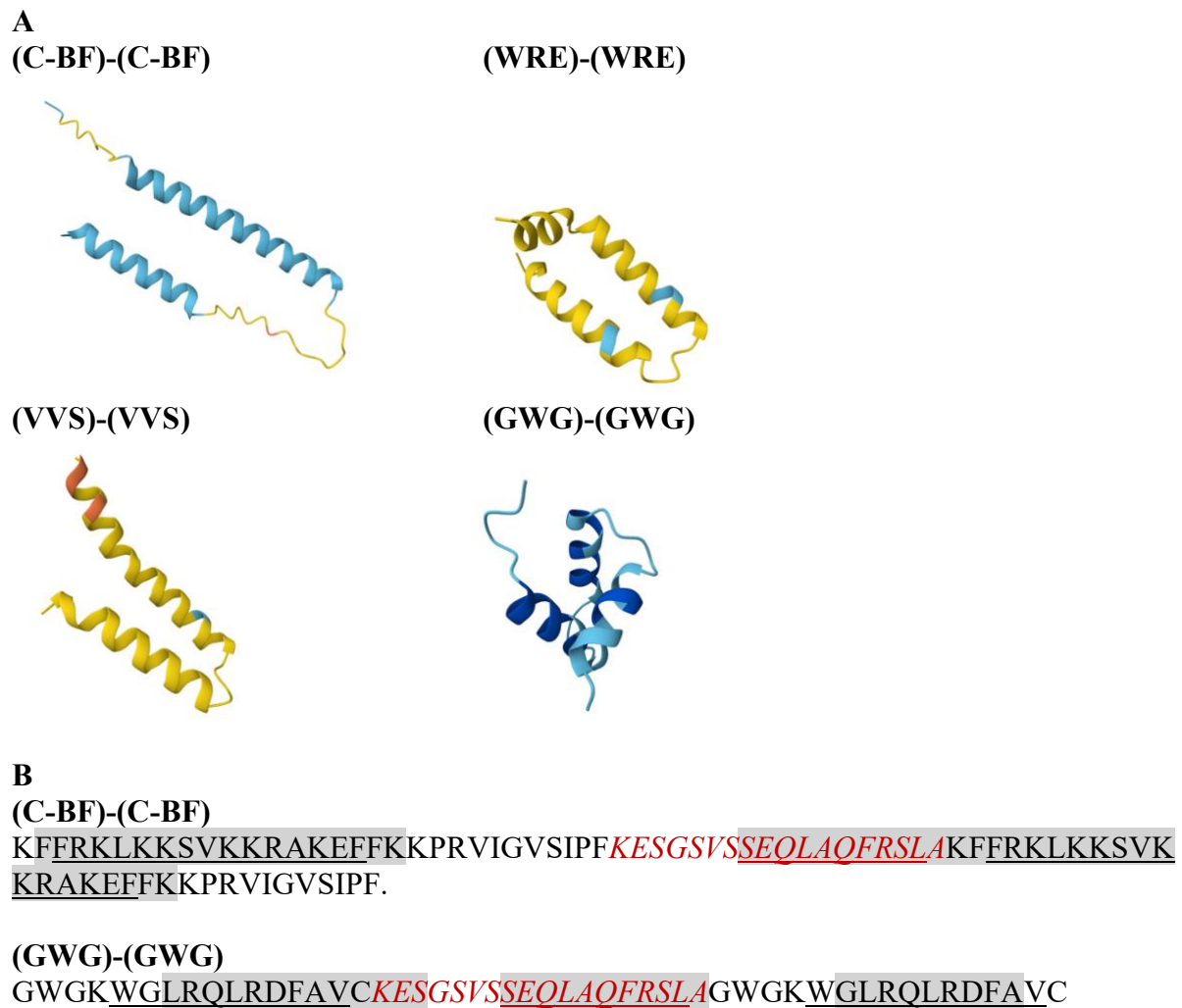
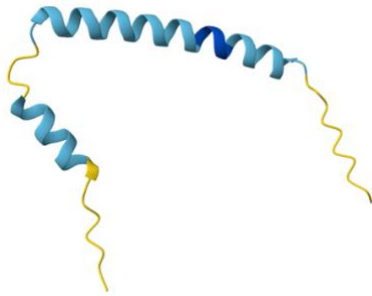


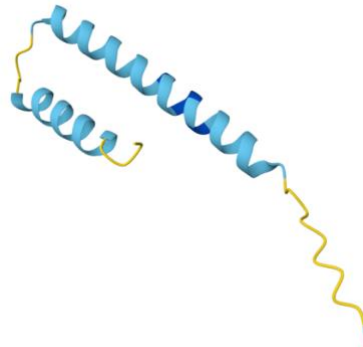
Figure S4. AlphaFold 3 structure predictions for AMP homodimers. A. Secondary structure predictions are shown for each AMP. Confidence in predicted structure is shown by the colour of the structure: pLDDT - very high: >90, dark blue; confident: 70-90, light blue; low: 50-70, yellow. **B.** Amino acid sequences indicate regions of predicted alpha helices (shaded) in the homodimer compared to in the monomers or linker (underlined). Linker is shown in red text and italics.

A

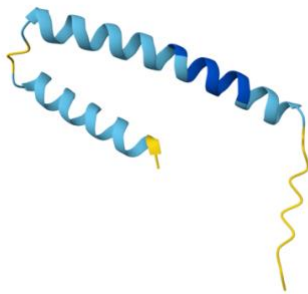
(WRE)-(C-BF)



(VVS)-(C-BF)



(GWG)-(C-BF)



B

(WRE)-(C-BF)

WRERLSAPGCFLAYLL*KESGSVS*SEQLAQFRSLAKFFRKLKKS~~VKKRAKEFFKKPRVIG~~
VSIPF

(VSS)-(C-BF)

VVSSWPWLLAHVSCWIK*KESGSVS*SEQLAQFRSLAKFFRKLKKS~~VKKRAKEFFKKPRVI~~
GVSIPF

(WGW)-(C-BF)

GWGKWGLRQLRDFAVC*KESGSVS*SEQLAQFRSLAKFFRKLKKS~~VKKRAKEFFKKPRV~~
IGVSIPF

Figure S5. AlphaFold 3 structure predictions for AMP heterodimers. A. Secondary structure predictions are shown for each AMP. Each Confidence in predicted structure is shown by the colour of the structure: pLDDT - very high: >90, dark blue; confident: 70-90, light blue; low: 50-70, yellow. **B.** Amino acid sequences indicate regions of predicted alpha helices (shaded) in the homodimer compared to in the monomers or linker (underlined). Linker is shown in red text and italics.

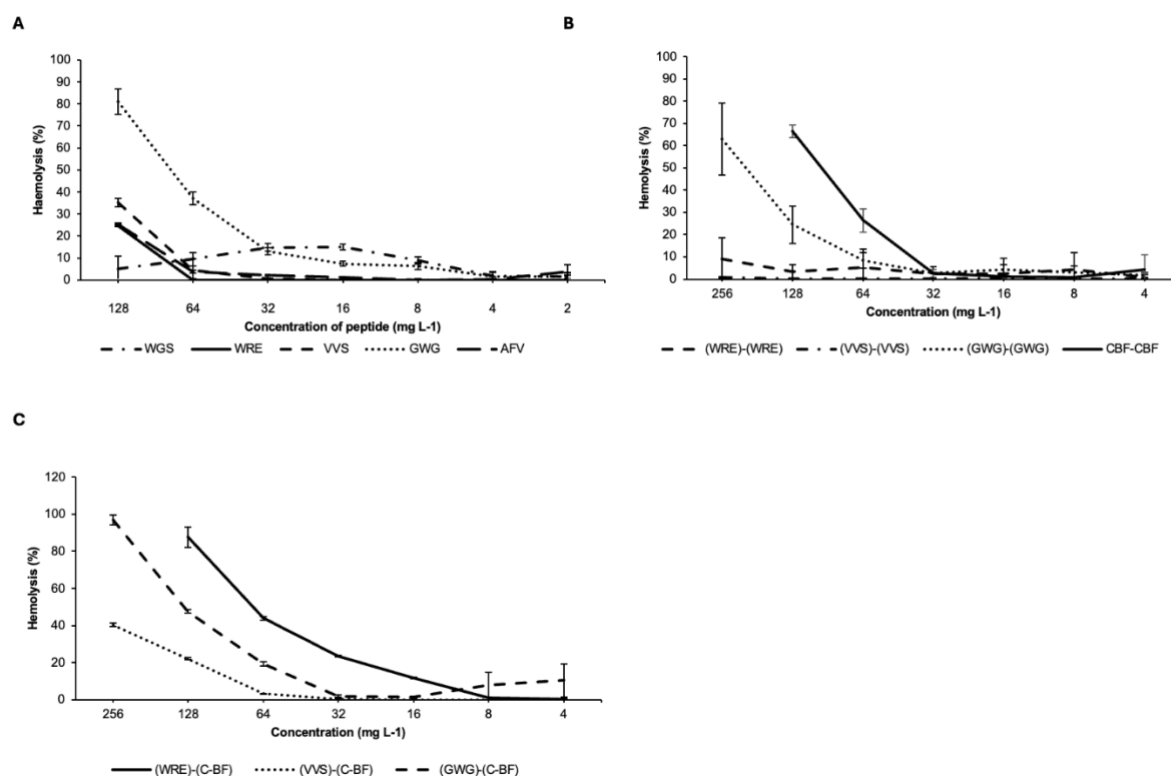


Figure S6. Haemolytic activity of AMPs. Each peptide was tested at the indicated concentration range by addition to an 8% (v/v) blood suspension and incubation for 1 hour at 37°C. Absorbance readings were taken of the supernatant at 414 nm and the percentage haemolysis of the red blood cells was calculated following the procedure reported by Oddo and Hansen [1]. **A.** monomer peptides; **B.** homodimer peptides; **C.** heterodimer peptides. The HC_{50} values were calculated in excel using the forecast function and the data either side of the peptide concentrations that resulted in 50% haemolysis.

SI References

1. Oddo A, Hansen PR. Hemolytic Activity of Antimicrobial Peptides. *Methods Mol Biol* 2017; 1548:427-35.