



*A novel antibacterial peptide derived from Crocodylus siamensis haemoglobin hydrolysate induces membrane permeabilization causing iron dysregulation, oxidative stress and bacterial death*

Article

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1 **A novel antibacterial peptide derived from *Crocodylus siamensis* hemoglobin hydrolysate induces membrane**  
2 **permeabilisation causing iron dysregulation, oxidative stress and bacterial death**

3

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24

25 **Abstract**

26 **Aims:** A novel antibacterial peptide from *Crocodylus siamensis* hemoglobin hydrolysate (CHHs) was characterised  
27 for antimicrobial activity.

28 **Methods and Results:** CHHs was hydrolysed for 2 h (2h-CHH), 4 h (4h-CHH), 6 h (6h-CHH) and 8 h (8h-CHH).  
29 8h-CHH showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and  
30 *Pseudomonas aeruginosa* at concentrations of 20, 20, 20 and 10 mg ml<sup>-1</sup> (w/v), respectively. Fluorescent  
31 microscopy revealed that 8h-CHH had bactericidal activity against *E. coli* and *P. aeruginosa*.  $\beta$ -galactosidase assay  
32 supported by RT-qPCR demonstrated that 8h-CHH resulted in differential expression of genes involved in iron  
33 homeostasis (*ftnA* and *bfd*) and oxidative stress (*sodA*, *soxR* and *oxyR*). Siderophore assay indicated that 8h-CHH  
34 also impaired siderophore production with diminished expression of *pvdF*. This pattern of gene expression suggests  
35 that 8h-CHH triggers the release of free ferric ions in the cytoplasm. However, decreased expression of genes  
36 associated with the SOS response (*recA* and *lexA*) in combination with neutral comet revealed that no DNA damage  
37 was caused by 8h-CHH. Membrane permeabilisation assay indicated that 8h-CHH caused membrane leakage  
38 thought to mediate the antibacterial and iron-stress responses observed, due to loss of regulated iron transport. The  
39 novel active peptide from 8h-CHH was determined as QAIHNEKVQAHGKKVL (QL17), with 41 %  
40 hydrophobicity and +2 net charge.

41 **Conclusions:** The QAIHNEKVQAHGKKVL fragment of *Crocodylus siamensis* hemoglobin is antibacterial via a  
42 mechanism that likely relies on iron dysregulation and oxidative stress which results in bacterial death.

43 **Significance and Impact of the Study:** We have described for the first time, a novel peptide derived from  
44 *Crocodylus siamensis* hemoglobin hydrolysate, that has the potential to be developed as a novel antimicrobial  
45 peptide.

46

#### 47 **Keywords**

48 *Crocodylus siamensis*, hemoglobin hydrolysate, antibacterial, peptide, oxidative stress genes, iron homeostasis  
49 genes.

50

#### 51 **Running headline**

52 Antibacterial peptide from *C. siamensis* hemoglobin

53

#### 54 **Introduction**

55 Bacterial infections account for a significant proportion of the global infectious disease burden, and morbidity and  
56 mortality rates caused by infectious microbial agents pose serious public health concerns. This is exacerbated by  
57 increasing resistance to antibiotics which is significant in an era where the development of new, synthetic  
58 antibacterial drugs lags the emergence of antimicrobial resistance (Mbah *et al.* 2012). It is therefore paramount to  
59 broaden the search for new antimicrobial substances, including the exploitation of novel sources where possible  
60 (Song *et al.* 2012). A growing area of research has begun to focus on protein hydrolysates of animal origin, such as  
61 goat whey protein hydrolyzed by treatment with alcalase, and which shows broad spectrum antibacterial activity  
62 (Osman *et al.* 2016). Similarly, an antibacterial peptide derived from acid extract of chicken (*Gallus gallus*) blood  
63 has efficacy against *E. coli* by a mechanism that results in toroidal pore formation and subsequent bacterial lysis  
64 (Vasilchenko *et al.* 2016). Furthermore, a peptic hemoglobin hydrolysate from bovine hemoglobin has also been  
65 shown to possess antibacterial activity (Froidevaux *et al.* 2001; Daoud *et al.* 2005; Arroume *et al.* 2006; Arroume *et*  
66 *al.* 2008; Adje *et al.* 2011). Whilst these areas of research are largely in their infancy, they provide a diverse, yet  
67 novel, means of informing the development of new peptide-based antimicrobial treatments.

68 *Crocodylus siamensis* is a small, freshwater crocodilian. In the wild these crocodiles experience many  
69 traumatic wounds that might be expected to be rife with infection from endogenous environmental bacteria, but this  
70 is not seen to be the case. *C. siamensis* (Siamese crocodile) hemoglobin constitutes the most abundant component in  
71 crocodile blood and has been long associated with a broad spectrum of biological activity, including antimicrobial  
72 (Srihongthong *et al.* 2012; Pakdeesuwan *et al.* 2017), antioxidant (Jandaruang *et al.* 2012; Srihongthong *et al.* 2012;  
73 Phosri *et al.* 2014; Maijaroen *et al.* 2016; Pakdeesuwan *et al.* 2017; Phosri *et al.* 2017) and anti-inflammatory  
74 activity (Phosri *et al.* 2014; Jangpromma *et al.* 2017; Phosri *et al.* 2017). Antibacterial activity has been attributed to  
75 peptides derived from *C. siamensis* hemoglobin and they are currently thought to be targeted to the bacterial surface  
76 (Srihongthong *et al.* 2012; Pakdeesuwan *et al.* 2017). However detailed studies of antimicrobial activity, mechanism  
77 of action and critical peptide sequences that mediate the observed activity are currently lacking.

78 This study aimed to investigate the antibacterial activity of hemoglobin hydrolysate from *C. siamensis*  
79 blood (CHH), hydrolysed for 2, 4, 6 and 8 h with pepsin, and to identify specific antimicrobial peptide fragments.  
80 Minimum Inhibitory Concentration (MIC), Time-Killing kinetics and viability staining revealed both bactericidal  
81 and bacteriostatic activity of the crude, hydrolysed peptide cocktail. To establish a mechanism of activity, the effect  
82 of CHHs on bacterial iron homeostasis was investigated using *ftnA* and *bfd* linked reporter strains, and analysis of

83 siderophore production. Due to the close association of iron homeostasis and oxidative stress, the expression levels  
84 of oxidative stress response genes (*oxyR*, *sodA* and *soxR*) were also investigated but ruled out the inclusion of the  
85 SOS response genes or associated DNA damage as a mechanism of action. However, membrane permeability assay  
86 indicated the hemoglobin fragments disrupted the cell envelope; the active peptide was found to be positively  
87 charged and therefore likely functioned in a similar manner to cationic antimicrobial peptides.

88

## 89 **Materials and methods**

### 90 **Bacterial strains**

91 *Escherichia coli* (NCTC 10418), *Pseudomonas aeruginosa* (PAO1), *Staphylococcus aureus* (NCTC 13141),  
92 *Klebsiella pneumonia* (ATCC 13883), *Bacillus subtilis* were maintained on nutrient agar (NA) or nutrient broth  
93 (NB), aerobically at 37°C throughout the study, unless otherwise stated. *E. coli* MC4100, *E. coli* MC4100 *fnA-lacZ*,  
94 *E. coli* MC4100 *bfd-lacZ*, *E. coli* H1914  $\Delta$ *fur-ftnA-lacZ*, *E. coli* H1914  $\Delta$ *fur-bfd-lacZ* carried stable chromosomal  
95 mutations that allowed them to be maintained on NB or NA as described above; these strains were provided by Prof.  
96 Simon Andrews (University of Reading).

97

### 98 **Hemoglobin extraction**

99 The extraction of hemoglobin from red blood cells (RBCs) was performed following the method of Srihongthong *et*  
100 *al.* (2012). The RBCs were washed three times with phosphate buffered saline (PBS) pH 7.0 and centrifuged at 3000  
101  $\times g$  for 5 min at 4°C. Ice-cold distilled water with five-fold volume was added to the RBCs pellet, vigorously mixed  
102 and allowed to settle for 10 min. After centrifugation at 10000  $\times g$  for 20 min at 4°C the supernatant was collected,  
103 lyophilized and stored at -70°C.

104

### 105 **Enzymatic hydrolysis**

106 Enzymatic hydrolysis was performed according to the method of Yu *et al.* (2006). Briefly, the hemoglobin solution  
107 was digested with pepsin (at pH 2.0) using a ratio of enzyme to substrate (1:100 w/w) at 37°C for 2, 4, 6 and 8 h and  
108 boiled at 95°C for 10 min to inactivate the enzyme. The insoluble material was removed by centrifugation at 7168  $\times$   
109  $g$  for 20 min. The supernatant was collected and adjusted to pH 7.0 by addition of 1 M HCl or 1 M NaOH. Finally  
110 the supernatant (hydrolysate) was lyophilized and stored at -20°C.

111

112 **Minimum inhibitory concentration (MIC) and time –killing assay (TKA)**

113 The minimum inhibitory concentration (MIC) of CHHs was determined by microbroth dilution in a microtiter plate  
114 assay system using a total volume of 110  $\mu$ l per well. Each well contained 10  $\mu$ l bacterial suspension (*E. coli* NCTC  
115 10418, *S. aureus*, *K. pneumoniae* and *P. aeruginosa*; adjusted to OD 0.1  $A_{650}$ ) and 100  $\mu$ l of CHHs. Plates were  
116 incubated for 24 h (MIC assay) or 9 h (TKA) at 37°C. An end point reading was taken for MIC, and hourly readings  
117 were taken for TKA ( $A_{650}$ ) (Spectrostar<sup>Nano</sup>, BMG Labtech).

118

119 **Fluorescent microscopy using BacLight™ to assess bacterial viability**

120 Test microorganisms (*E. coli* NCTC 10418, *S. aureus*, *K. pneumoniae*, *B. subtilis* and *P. aeruginosa*) were incubated  
121 for 16 h with 10, or 20 mg ml<sup>-1</sup> CHHs (determined from MIC). Cultures were centrifuged at 9000  $\times$  g for 5 min and  
122 the supernatant was discarded. The cell pellets were re-suspended in 100  $\mu$ l dH<sub>2</sub>O containing SYTO9 and PI at a  
123 ratio of 2:1. Cells were incubated in the dark at room temperature for 45 min and 10  $\mu$ l transferred to a glass slide.  
124 Cells were visualized by fluorescent microscopy (Nikon eclipse 80i) using oil immersion and  $\times$ 100 lens. SYTO9  
125 detection (viable cells) was used a 488 nm excitation and 520 nm emission filter. Propidium iodine (PI) detection  
126 (non-viable cells) was used 543 nm excitation and 572 nm emission filter.

127

128  **$\beta$ -galactosidase assay**

129 Test microorganisms (*E. coli* MC4100, *E. coli* MC4100 *ftnA-lacZ*, *E. coli* MC4100 *bfd-lacZ*, *E. coli* H1914  $\Delta$ *fur*-  
130 *ftnA-lacZ*, *E. coli* H1914 $\Delta$ *fur-bfd-lacZ*) were cultured in 100  $\mu$ l NB in a 96-well microtiter plate until mid-log was  
131 reached. Then 100  $\mu$ l of 2, 4, 6 and 8h-CHH (20 mg ml<sup>-1</sup>) was added to each well followed by an additional  
132 incubation for 1.5 h at 37°C. Bacteria were permeabilised with buffer containing 60 mM Na<sub>2</sub>HPO<sub>4</sub>·7 H<sub>2</sub>O, 40 mM  
133 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 10 mM KCl, 1 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 50 mM  $\beta$ -mercaptoethanol, 0.1 % SDS and 4  $\mu$ l of chloroform  
134 for 5 min. Bacterial lysate was transferred to a fresh MTP and 20  $\mu$ l of ONPG was added to each sample. The plate  
135 was incubated in the dark at 37°C for 10 min, and absorbance values were read at 420 nm and 550 nm  
136 (Spectrostar<sup>Nano</sup>, BMG Labtech). Miller units were calculated based on the following formula (Miller 1972):

137 
$$\text{Miller Units} = 1,000 \times [\text{OD}_{420} - (1.75 \times \text{OD}_{550})] / [T \times V \times \text{OD}_{600}]$$

138 where T determines the reaction time in minute and V determines the volume of cultured assayed in ml.

139

#### 140 **CAS agarose diffusion (CASD) assay**

141 CAS agarose diffusion assay followed the method of Schwyn and Neilands (1987). Pre-cultures of (*E. coli* MC4100,  
142 *E. coli* NCTC 10418, *S. aureus*, *K. pneumoniae*, *B. subtilis* and *P. aeruginosa*) were equilibrated to OD 0.1 ( $A_{650}$ )  
143 and supplemented with 20 mg ml<sup>-1</sup> of 2, 4, 6 and 8h-CHH and 1 mM DTPA. Treated cultures were incubated for a  
144 further 24 h at 37°C. After centrifuging at 9000 × *g* for 3 min (Rotina 380R centrifuge; Hettich, Germany) 50 μl  
145 aliquots of the sample supernatant were added to wells bored into the center of Petri dishes filled with 25 ml CAS  
146 agarose agar. After incubating in the dark at room temperature for 2 h, digital calipers were used to measure the  
147 diameter (mm) of the yellow diffusion zone diameters which is an indicator of siderophore production and the  
148 reduction of ferric iron.

149

#### 150 **RNA extraction and cDNA synthesis**

151 Total RNA was extracted from bacterial cells per the manufacturer's instructions using the SV Total RNA extraction  
152 kit (Promega, USA). Total RNA concentration and purity were determined using Nanodrop ND-1000  
153 spectrophotometer (Nanodrop Technologies, USA). RNA samples with an absorbance ratio at OD 260/280 between  
154 1.8-2.2 and OD 260/230 < 2.0 were used for further analysis. For each sample, cDNA was synthesized using the  
155 High-Capacity cDNA Reverse Transcription kit according to the manufacturer's instructions (Applied Biosystem  
156 Inc).

157

#### 158 **Reverse transcription - real time PCR (RT-qPCR) assay**

159 Primers used in this study are shown in Table 1. All PCR reactions were performed using 7500 Fast Real-Time PCR  
160 System machine under following conditions: 15 min at 95°C and 40 cycles of 3 s at 95°C, 30 s at 68°C in 96-well  
161 clear optical reaction plates (Applied Biosystem, USA). The procedure ended by melt-curve ramping from 60 to  
162 95°C for 20 min to check the PCR specificity. All RT-qPCR reactions were carried out in biological and technical  
163 triplicate. A non-template control was also included in each run for each gene.

164

#### 165 **Neutral comet assay**



166 Neutral comet assay was determined following the method of Solanky and Haydel (2012) with some modifications.  
167 Frosted microscope glass slides with a clear window were pre-coated by dipping in 1% agarose solution prepared  
168 with sterile water and were dried in an incubator at 40°C for 30 min. *E. coli* MC4100 cultures were incubated with  
169 rotary agitation at 37°C until logarithmic phase of growth (OD ~0.1; A<sub>600</sub>), and then diluted with NB broth to  
170 achieve a concentration of ~10<sup>7</sup> CFU ml<sup>-1</sup>. The cells were collected by centrifugation (9000 × g, 15 min), washed  
171 with 0.1× phosphate-buffered saline (PBS), and re-suspended in one of the following solutions: 1% TritonX-100; 5  
172 mM H<sub>2</sub>O<sub>2</sub>; deionized H<sub>2</sub>O and 8h-CHH 20 mg ml<sup>-1</sup>. Exposed cells were then incubated for 30 min at 37°C.  
173 After incubating the slide at 4°C for 10 min to allow the initial layer of agarose to cool, the coverslip was removed  
174 and a subsequent agarose layer was added. The first layer consisted of 200 μl of 0.5% agarose prepared in 0.1× PBS  
175 and maintained at 55 –60°C for 30 min. For the second layer, 2 μl of exposed cells was mixed thoroughly with 200  
176 μl of 0.5% agarose solution and 100 μl of this mixture was transferred to the slide. A third layer was consisted of 5  
177 μg ml<sup>-1</sup> RNaseA and 1 mg ml<sup>-1</sup> lysozyme in 0.5% agarose solution. Slides were refrigerated for 10 min at 4°C and  
178 incubated for 30 min at 37°C. Embedded cells were then lysed by immersing slides in a solution containing 2.5 M  
179 NaCl, 100 mM EDTA, 10 mM Tris pH 10 and 1% TritonX-100 for 1 h at room temperature. Following lysis, slides  
180 were immersed in an enzyme digestion solution prepared with 2.5 M NaCl, 10 mM EDTA, 10 mM Tris pH 7.4, and  
181 0.5 mg ml<sup>-1</sup> of proteinase K for 2 h at 37°C. Lysis and enzyme digestion steps were carried out in the dark to prevent  
182 light exposure. Slides were immersed in buffer containing 300 mM sodium acetate and 100 mM Tris, pH 9 for 20  
183 min. Slides were electrophoresed at 12 V for 50 min. Following electrophoresis, slides were sequentially immersed  
184 in 1 M ammonium acetate prepared in ethanol for 20 min and 75% ethanol for another 30 min. Slides were then  
185 allowed to dry. Prior to staining, slides were pretreated with a freshly prepared solution of 5% DMSO and 10 mM  
186 NaH<sub>2</sub>PO<sub>4</sub>. While the slides were still wet, DNA was stained with 50 μl of 1 μM YOYO-1 in 5% DMSO and  
187 visualized using a Nikon eclipse 80i fluorescent microscope at 100× magnification with the appropriate filter set for  
188 YOYO-1 (excitation 491 nm and emission 509 nm). Comets were imaged and comet lengths were measured using  
189 Volocity software version 5.5.

190

### 191 **Cytoplasmic membrane permeability assay**

192 Cytoplasmic membrane permeability assay was determined following the method of Chitemerere and  
193 Mukanganyama (2014) with modifications. Briefly, bacteria (*E. coli* MC4100, *P. aeruginosa*, *S. aureus*) were grown

194 to mid-exponential phase (OD 0.2-0.4; A<sub>650</sub>); 2 ml were mixed with an equivalent volume of 1 μM diSC3-5 dyes  
195 and incubated 1 h, for maximal uptake of dye, then were collected by centrifugation (3000 × g for 5 min). Cells were  
196 wash and re-suspended in 2 ml of buffer (5 mM HEPES, pH 7.2, 5 mM glucose) and the absorbance was measured  
197 at an excitation wavelength of 622 nm and emission wavelength of 670 nm. Afterward, 2 ml of 100 mM KCl was  
198 added to equilibrate the cytoplasmic and external K<sup>+</sup> ion concentrations. Cells were mixed with and equal volume of  
199 sample then the fluorescence was monitored at an excitation wavelength of 622 nm and emission wavelength of 670  
200 nm at 1 h intervals. Dye released with de-ionised water was used as a negative control.

201

### 202 **Amino acid sequence analysis**

203 The active fraction of 8h-CHH was selected and the contained peptides identified using LTQ Orbitrap XL Mass  
204 spectrometry employing the following search parameters: non-specified enzymatic cleavage with three possible  
205 missed cleavages, +/-0.8 Da mass tolerances for MS and MS/MS, a peptide mass tolerance of +/-5 ppm,  
206 methionine oxidation and Gln->pyro-Glu (N-term Q) variable modification and monoisotopic mass. Data were  
207 additionally processed at the Mascot Server (<http://www.matrixscience.com/>) using MS/MS ion searches against  
208 SwissProt (current release).

209

### 210 **Statistical analysis**

211 Statistical analysis was performed using ANOVA and followed by Dunnett's test. Data are presented as mean ±  
212 SEM. A value of  $P < 0.05$  was accepted to be significant ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ).

213

## 214 **Results**

### 215 **Bacteriostatic and bactericidal effects of hemoglobin hydrolysate**

216 Bacteriostatic and bactericidal effects of hemoglobin hydrolysate were investigated by minimum inhibitory  
217 concentration (MIC), time-killing assay (TKA) and fluorescent microscopy using BacLight™. The MIC was  
218 determined using 2h-CHH, 4h-CHH, 6h-CHH and 8h-CHH, and results are presented in Table 2. Gram-negative  
219 microorganisms generally required higher concentrations of hydrolysed CHHs to inhibit growth. The results showed  
220 that all hydrolysed CHHs were antibacterial at concentrations of 20 mg ml<sup>-1</sup> (w/v) against *E. coli* and *S. aureus*.  
221 Furthermore, there was antibacterial activity for all CHHs at concentrations of 10 mg ml<sup>-1</sup> (w/v) against *K.*

222 *pneumoniae* and *P. aeruginosa*. Significantly, the dose required to inhibit growth decreased as the length of time  
223 that the hemoglobin was hydrolysed increased, suggesting that higher concentrations of shorter peptide fragments  
224 mediated the inhibitory activity. This was verified by TKA which indicated that 8h-CHH resulted in the highest  
225 percentage of bacterial death (Table 3). Samples analysed by BacLight™ viability staining and fluorescent  
226 microscopy confirmed that 8h-CHH at a dose of 20 and 10 mg ml<sup>-1</sup> caused bacterial death (*E. coli* NCTC 10418 and  
227 *P. aeruginosa*; Fig. 1a and 1b, respectively). At lower doses and shorter hydrolyze times, CHH treatments tended to  
228 be bacteriostatic rather than bactericidal. Both mechanisms were investigated as described below.

229

### 230 **Hemoglobin hydrolysate alters bacterial iron homeostasis and causes oxidative stress without inducing** 231 **irreversible DNA-damage or the SOS response**

232 The  $\beta$ -galactosidase assay demonstrated that 2h-CHH, 4h-CHH, 6h-CHH and 8h-CHH at 20 mg ml<sup>-1</sup> decreased the  
233 expression of *ftnA* while increasing the expression of *bfd*, under iron rich conditions (Fig. 2). Decreased expression  
234 of *ftnA* is indicative of iron restriction whereas increased expression of *bfd* is affiliated with iron repletion and is  
235 known to have a role in haem-iron handling. The expression profile was not altered in a  $\Delta fur$  background suggesting  
236 that the dysregulated expression of *ftnA* and *bfd* was not the result of Fur-dependent iron-mediated regulation (Fig.  
237 2). However, CASD assay indicated that CHHs decreased the production of siderophores (Fig. 3) and following  
238 treatment with 8h-CHH, the expression of *pvdF* (associated with siderophore synthesis) also significantly decreased  
239 ( $P < 0.05$ ) (Fig. 4b) suggesting some degree of altered iron homeostasis.

240 Bacterial iron metabolism and oxidative stress are inextricably linked; RT-qPCR demonstrated that  
241 following treatment with 8h-CHH, the expression of oxidative stress response genes *sodA* and *soxR* significantly  
242 increased while the expression of *oxyR* decreased (Fig. 4a). Oxidative stress is correlated with DNA damage and  
243 subsequent induction of the SOS system of repair. However, the expression of *recA* was diminished and *lexA*  
244 unchanged following treatment with 8h-CHH suggesting that the SOS response was not initiated (Fig. 4c).  
245 Therefore, the dysregulated iron homeostasis observed might be mediated by transcriptional responses associated  
246 with oxidative stress, rather than in response to environmental iron availability.

247

### 248 **Hemoglobin hydrolysate does not cause DNA damage**

249 Neutral Comet Assay verified that CHH did not cause DNA damage. Treatment with 1% TritonX-100 and 5 mM  
250 H<sub>2</sub>O<sub>2</sub> (positive controls) produced an increase in comet length, and therefore DNA degradation, relative to the  
251 negative control (DI sterilized water). Exposure of *E. coli* MC4100 to 1% TritonX-100 and 5 mM H<sub>2</sub>O<sub>2</sub> for 30 min  
252 increased the comet length values of 49.79  $\mu$ m and 21.12  $\mu$ m, respectively. Meanwhile the exposure of 8h-CHH  
253 resulted in comet length values of 11.24  $\mu$ m and the negative control, 10.78  $\mu$ m as shown in Table 4. The 1%  
254 TritonX-100 also yielded higher a comet length value, demonstrating higher DNA double strand break (DSB) levels  
255 while DNA double strand break wasn't caused by 8h-CHH upon exposure in bacterial cells.

256

### 257 **Hemoglobin hydrolysate causes bacterial membrane leakage**

258 Many short peptides aggregate in bacterial membranes resulting in a loss of integrity, such loss of integrity allows  
259 influx and efflux of various solutes and ions, including metal ions such as iron. It was hypothesized that membrane  
260 disruption might underpin the iron homeostatic dysregulation and concurrent oxidative stress response described  
261 above. Indeed, results from cytoplasmic membrane permeability assay, showed a gradual increase of diSC3-5 dye  
262 release over time in the presence of 8h-CHH as well as in the presence of the 1% TritonX-100 positive control (Fig.  
263 5a, 5b and 5c). Similarly, diSC3-5 was released from all test microorganisms following exposure to 8h-CHH for 60  
264 min (Fig. 5). The effect was most marked for the Gram-negative test microorganisms (*E. coli* and *P. aeruginosa*)  
265 indicating that 8h-CHH can permeabilise the cell envelope more effectively than that of Gram-positive  
266 microorganisms (*S. aureus*).

267

### 268 **Active peptide was identified by LTQ Orbitrap XL mass spectrometry**

269 To establish the identity of the active CHH peptide, the primary sequence was determined using LTQ Orbitrap XL  
270 mass spectrometry. As the most active CHH, the amino acid sequences of 8h-CHH was determined as  
271 QAIHNEKVQAHGKKVL (QL17) corresponding to a molecular mass of 1895.07 Da. The obtained sequence was  
272 uploaded to the antimicrobial database (<http://aps.unmc.edu/AP/main.php>) and the protein databank  
273 (<https://www.ncbi.nlm.nih.gov>) for further characterization. As shown in Table 5, antibacterial peptide QL 17 had  
274 hydrophobicity values of approximately 41%, with a net charge of +2. Alignment of the amino acid sequences of the  
275 peptide fragments with *C. siamensis* hemoglobin indicated the antibacterial peptide originated from the  $\beta$ -subunit of

276 *C. siamensis* hemoglobin. The short length and positive charge of the peptide indicates that it might aggregate in the  
277 bacterial cell envelope in much the same way as cationic antimicrobial peptides.

278

## 279 **Discussion**

280 Protein hydrolysates are gaining popularity for their potential therapeutic effects due to demonstrable efficacy and  
281 low toxicity. This study used MIC, time-killing kinetics and viability staining to ascribe antimicrobial activity to  
282 hemoglobin hydrolysate from *C. siamensis*. CHHs were either bacteriostatic or bactericidal depending on the species  
283 of bacteria, dose and length of hydrolyzed time. Longer hydrolysis times correlated with higher inhibition of growth,  
284 with 8h-CHH proving to be the most efficacious. The antibacterial mechanism was investigated with focus on iron  
285 homeostasis and oxidative stress which in numerous pathogens is associated with host haem-iron availability.

286 The expression of *ftnA* was increased and that of both *bfd* and *pvdF* decreased following exposure to 8h-  
287 CHH. Under iron repleted conditions expression of *ftnA* and *bfd* is ordinarily repressed via a process relying on the  
288 ferric uptake regulator (Fur). The observed expression profile was no concordant with typical iron-Fur regulation  
289 which was verified using a  $\Delta fur$  background, which showed the same expression profile as the wild-type  
290 background. However, differential expression of *ftnA*, *bfd* and *pvdF* also occurs in response to oxidative stress,  
291 which is closely allied to iron homeostasis (Zheng *et al.* 1999). Quantitative analysis of the expression of *soxR* and  
292 *oxyR* indicated differential expression in response to CHH-treatment akin to that observed during oxidative stress,  
293 indicating that CHH induced a state of oxidative stress in the microorganisms tested.

294 Uncontrolled oxidative stress results in widespread lipid, protein and DNA damage (Gault *et al.* 2016). If  
295 DNA damage becomes too great, the bacterial SOS system is activated. *LexA* is the master regulator of the SOS  
296 response; under normal conditions it represses the expression of genes encoding several DNA repair proteins,  
297 including *recA*. *LexA* has a negative auto-regulatory function and when the SOS response is triggered, increased  
298 levels of *lexA* are also produced which ultimately serves as a negative feedback mechanism to switch of the SOS  
299 response once rescue is achieved (Michel 2005). In this study, the expression of *lexA* was unchanged and *recA*  
300 decreased following treatment with CHH indicating that while oxidative stress occurred it was below the threshold  
301 to necessary to induce the SOS system. This was verified by the absence of DNA damage (a key signal for SOS  
302 induction) observed by neutral comet assay.

303           The bacterial response to outer membrane stress is distinct to the oxidative stress and SOS response, but  
304 triggers several stress-associated, damage repair pathways to maintain membrane integrity and prevent influx/efflux  
305 between the cytoplasm and external environment. Cationic antimicrobial peptides are well known to disrupt the  
306 bacterial cell envelope by aggregating to form pores within the membrane. Ordinarily this causes catastrophic  
307 damage resulting in bacterial death by lysis. Analysis of membrane integrity following treatment with CHH  
308 indicated that 8h-CHH could permeabilise *E. coli*, *P. aeruginosa* and *S. aureus* (Chitemerere and Mukanganyama  
309 2014). The active peptide from 8h-CHH was identified using LTQ-Orbitrap XL mass spectrometry and the sequence  
310 determined as QAIHNEKVQAHGKKVL (QL17) corresponding to a molecular mass of 1895.07 Da. The peptide  
311 QL17 had hydrophobicity values of about 41%, together with a net charge of +2. Given its positive charge, this  
312 fragment is hypothesized to aggregate within the bacterial membrane, in the same way as cationic antimicrobial  
313 peptides (Sato and Feix 2006). It is known that antimicrobial peptides positive charge combined with  
314 hydrophobicity is critical for partitioning of the peptide into the bacterial cell membrane (Pata *et al.* 2011).

315           Alignment the amino acid sequences of the peptide fragment with *C. siamensis* hemoglobin indicated the  
316 antibacterial peptide originated from the  $\beta$ -subunit of *C. siamensis* hemoglobin. This result agrees with Arroume *et*  
317 *al.* (2008), who reported that antimicrobial products derived from hemoglobin hydrolysis are cleaved from the  $\alpha$ -  
318 and  $\beta$ -subunits. These products mostly consist of ~15–30 amino acid residues and have a molecular weight < 10  
319 kDa. Of note, was the correlation between length of hydrolysis time and antimicrobial activity. As expected,  
320 hemoglobin hydrolysed for longer periods of time comprised a higher concentration of small peptide fragments,  
321 which in turn exhibited better antimicrobial efficacy, highlighting the importance of peptide fragment size for  
322 activity.

323           Taken collectively, the data derived from this study indicates that the hemoglobin of *C. siamensis* can be  
324 hydrolyzed to produce a novel antimicrobial peptide that at high concentrations mediates bacterial death by  
325 aggregating in the cell envelope and damaging membrane integrity. The consequent influx of exogenous material  
326 combined with an efflux of cytoplasmic content likely underpins the dysregulation of iron homeostasis and  
327 concurrent oxidative stress. Whilst the potential to utilise antimicrobial peptides derived from hemoglobin warrants  
328 further study, blood derived from *C. siamensis* is currently a waste-product of farming. Therefore, with larger  
329 studies drawn from this exploratory research, it could instead be developed as a natural or synthetic antimicrobial  
330 peptide, engineered to ensure maximum efficacy and minimal toxicity.

331

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342

### 343 **Conflict of Interest**

344 The authors confirm that this article content has no conflicts of interest.

345

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414

415 **Table 1** Primer pairs for RT-qPCR

416

Species	Gene names	GeneBank accession no.	Region	Product size (bp)	Forward primer (5'→3')	Reverse primer (5'→3')
<i>E. coli</i>	<i>oxyR</i>	HG738867	2620691..2621608	197	CCCCGGCTTCAAAACAGAAA	GCTGGTGAAAGAGAGCGAAG
	<i>sodA</i>		2678668..2679288	187	CGAAGTCACGTTTCGATAGCC	CTGCCAGAATTTGCCAACCT
	<i>soxR</i>		4163425..4163889	172	CAGCGGCGATATAAACGTGA	CCAACTCTTCTCGCCATTGG
	<i>rec A</i>		3960012..3961073	173	GAAGAACAAAATCGTGTGCGC	CATTTCGCTTTACCTGACCG
	<i>lex A</i>		4143071..4143679	158	GCAGGAAGAGGAAGAAGGGT	CTTTCATCGACATCCCCTG
<i>P. aeruginosa</i>	<i>rpoD</i>	BA000007	3952578..3954419	220	TTCGTACGCAAGAACGTCTG	AGGCCGGTTTCTTCTTCAAT
	<i>pvdF</i>	NC_002516	2652230..2653057	181	CGTACCAGCTCATCGAGGAT	AGACCCTGAACGACCTCTTG
	<i>rpoD</i>		634371..636224	186	GGGTCACATCGAACTGCTTG	TCATCGAGGACTCCACCATG

424

425 **Table 2** The MIC values of sample CHHs

426

Sample	MIC values (mg ml <sup>-1</sup> )				% Growth inhibition			
	<i>E.coli</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>	<i>E.coli</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
2h-CHH	20	20	10	20	33.33***±0.003	57.14***±0.015	34.11***±0.004	38.87***±0.005
4h-CHH	20	10	10	10	21.74***±0.025	34.29***±0.002	25.10***±0.002	21.11***±0.031
6h-CHH	20	10	10	20	37.86***±0.007	45.14***±0.008	35.03***±0.008	25.53***±0.020
8h-CHH	20	20	10	20	24.64***±0.003	41.10***±0.113	44.61***±0.020	31.20***±0.195

431

432 \*\*\* denotes  $P < 0.001$ . Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

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434

435 **Table 3** Time-killing assay (TKA) of sample CHHs

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Sample	%Growth inhibition at 9 h intervals			
	<i>E.coli</i>	<i>K.pneumoniae</i>	<i>P.aeruginosa</i>	<i>S.aureus</i>
2h-CHH	48.07***±0.001	3.21***±0.011	6.07***±0.004	31.23***±0.006
4h-CHH	55.64***±0.007	4.06***±0.002	4.98***±0.007	56.31***±0.007
6h-CHH	73.37***±0.001	0.06±0.002	10.65***±0.011	33.23***±0.013
8h-CHH	48.54***±0.020	12.00***±0.015	21.73***±0.004	17.88***±0.115

443

444 \*\*\* denotes  $P < 0.001$ . Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

445

446 **Table 4** Neutral bacterial comet assay following *E.coli* exposure to 1 % TritonX-100, 5 mM H<sub>2</sub>O<sub>2</sub>, 8h-CHH and DI water.

447

Comet length values (μm)	<i>E.coli</i> treated with			
	1% (v/v) TritonX-100	5 mM H <sub>2</sub> O <sub>2</sub>	8h-CHH	DI water
	49.79***±0.002	21.12***±0.010	11.24±0.014	10.78±0.009

450

451

452 \*\*\* denotes  $P < 0.001$ . Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

453

454 **Table 5** Structural characteristics of the antibacterial peptide from 8h-CHH

Property	Peptide sequence	Hydrolysate	%Hydrophobicity	Net charge	Sequence alignment
Antibacterial	QAIHHNEKVVQAHGKKVL (QL17)	8h-CHH	41%	+2	<i>C. siamensis</i> Hb β-subunit (position 53-69)

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477 **Figure captions**

478 **Figure 1** Representative images of Live and Dead staining assay (BacLight™ Fluorescent microscopy), red coloured  
479 cells are dead cells and green coloured cells are live cells, showed the bactericidal effect of 8h-CHH at a dose of 20  
480 and 10 mg ml<sup>-1</sup> against (a) *E.coli* and (b) *P.aeruginosa* PAO1, respectively.

481

482 **Figure 2** β- galactosidase assay. Expression of *ftnA* and *bfd* in response to treatment with CHHs does not exhibit  
483 iron-dependent expression in a wild-type or  $\Delta fur$  background. Each bar represents the mean  $\pm$  SEM (n=3). \*\*\*  
484 significant at  $P < 0.001$ .

485

486 **Figure 3** The effect of CHHs on siderophore production by CASD assay. Sample CHHs decreased the production of  
487 siderophore from all of bacterial strains over a time frame of 2 h. Each bar represents the mean  $\pm$  SEM (n=3). \*\*\*  
488 significant at  $P < 0.001$ .

489

490 **Figure 4** Effect of 8h-CHH on gene expression levels in *E.coli* and *P.aeruginosa* were analyzed by real time PCR  
491 (RT-qPCR). (a) A fold change gene expression of *oxyR*, *sodA* and *soxR* were validated for oxidative stress response.  
492 (b) A fold change gene expression of *pvdF* was validated for siderophore production and (c) a fold change gene  
493 expression of *recA* and *lexA* were validated for DNA damage (SOS response). Each bar represents the mean  $\pm$  SEM  
494 (n=3). \*significant at  $P < 0.05$ , \*\*significant at  $P < 0.01$  and \*\*\* significant at  $P < 0.001$ .

495

496 **Figure 5** Measurement of 3'3 dipropylthiadicarbocyanine (diSC3-5) dye release overtime from (a) *E.coli*, (b)  
497 *P.aeruginosa* PAO1 and (c) *S.aureus* membranes in the presence of 8h-CHH, permeabilizing agent 1% TritonX-100  
498 and negative control Deionize water (DI).

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