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**Identification of new anti-microbial peptides that contribute to the bactericidal activity of egg white against *Salmonella enterica* serovar Enteritidis at 45 °C**

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1 **Abstract**

2 A recent work revealed that egg white (EW) at 45 °C exhibits a powerful bactericidal activity  
3 against *S. enterica* serovar Enteritidis, which is surprisingly little affected by removal of the  
4 >10 kDa EW proteins. Here, we sought to identify the major EW factors responsible for this  
5 bactericidal activity by fractionating EW using ultrafiltration and nanofiltration, and by  
6 characterizing the physicochemical and antimicrobial properties of the resulting fractions. In  
7 particular, 22 peptides were identified by nano-LC/MS-MS and the bactericidal activities of  
8 representative peptides (with predicted antimicrobial activity) were further assessed. Two  
9 peptides (FVPPVQR and GDPSAWSWGAEAHS) were found to be bactericidal against *S.*  
10 *enterica* serovar Enteritidis at 45 °C when provided in an EW environment. Nevertheless, these  
11 peptides contribute only part of this bactericidal activity, suggesting other, yet to be determined,  
12 anti-microbial factors.

13 **Keywords:** egg white; ultrafiltration; antimicrobial activity; peptide; *Salmonella*  
14 Enteritidis

15

## 16 **Introduction**

17 Egg white (EW) represents a hostile medium for microorganisms due to its alkaline pH, high  
18 viscosity, nutrient deficiency and the array of antimicrobial proteins and peptides it contains (in  
19 particular lysozyme, ovotransferrin, protease inhibitors and vitamin-binding proteins)<sup>1,2</sup> .  
20 Lysozyme exerts a hydrolytic activity against the cell wall of Gram-positive bacteria leading to  
21 membrane disruption. Ovotransferrin is a high-affinity iron-chelating protein that promotes iron  
22 restriction and mediates damage to bacterial cytoplasmic membranes<sup>3</sup>. Protease inhibitors (e.g.  
23 ovomucoid, ovoinhibitor, cystatin and ovostatin) would inhibit proteases of pathogenic bacteria  
24 required for host colonization. EW vitamin-binding proteins, namely flavoprotein, avidin and  
25 the thiamine-binding protein sequester riboflavin, biotin and thiamine, respectively, and thus  
26 would induce a bacteriostatic effect. In addition, some minor proteins and peptides recently  
27 revealed by high-throughput approaches may also play a role in defence against bacterial  
28 contamination and it is quite possible that the various anti-bacterial factors associated with EW  
29 interact synergistically to enhance protection against bacterial invaders<sup>4</sup>.

30 Previous studies on the antimicrobial activity of chicken EW largely focused on *Salmonella*  
31 *enterica* serovar Enteritidis, hereinafter referred to as *S. Enteritidis*, since this serotype is the  
32 major food-borne pathogen (90%) associated with the consumption of eggs and egg products<sup>5</sup>.  
33 The high association of *S. Enteritidis* in egg-related salmonellosis is thought to be due to its  
34 specialized ability to survive exposure to the hostile conditions of EW<sup>6-8</sup>. It is generally  
35 accepted that upon exposure to EW *Salmonella* suffers from two major harmful influences, iron  
36 deficiency (resulting in a bacteriostatic effect) and cell-envelop damage (which is bactericidal)<sup>4</sup>.  
37 However, physicochemical factors, such as alkaline pH and temperature of incubation also play  
38 important roles in EW antimicrobial activity. Indeed, *S. Enteritidis* is able to grow weakly in  
39 EW at 20 °C and 30 °C<sup>2,9</sup>. However, at higher temperature ( $\geq 42$  °C), EW exerts a bactericidal  
40 effect against *S. Enteritidis*<sup>1,6,10</sup>. It is notable that the lowest temperature at which significant

41 bactericidal activity is observed for EW is close to that naturally encountered during egg  
42 formation (i.e. that of the hen body, 42 °C). For this reason, this temperature is routinely used  
43 in studies on the bactericidal activity of EW<sup>11,12</sup>. The importance of temperature in the  
44 antimicrobial activity of EW is highlighted by a method for pasteurisation of liquid EW  
45 involving heat treatment at 42-45 °C for 1 to 5 days. This treatment allows subsequent storage  
46 of EW at room temperature for several months<sup>13</sup> and, critically, it provides a complete killing  
47 of *S. Enteritidis* and is more efficient than the traditional EW pasteurization treatment (57 °C  
48 for 2 to 5 min) that requires subsequent storage under refrigeration.

49 Exposure of *S. Enteritidis* to EW model medium (namely egg white 10kDa filtrate  
50 supplemented with 10% EW) at 45 °C for 45 min results in extensive changes in global-gene  
51 expression<sup>10</sup> indicative of a major response of *S. Enteritidis* to nutrient deprivation (iron and  
52 biotin) and cell damage/stress, and a shift in energy metabolism and catabolism. These changes  
53 were considered to reflect attempts by *S. Enteritidis* to overcome the antibacterial activities of  
54 EW that lead to eventual cell death after prolonged incubation at 45 °C. Surprisingly, removal  
55 of the  $\geq 10$  kDa proteins from EW by ultrafiltration had little impact on the global expression  
56 pattern (only 64 genes were affected after 45 min, 2% of the total genome) and the bactericidal  
57 activity (over 24 h) when compared to the EW model medium, indicating that the EW proteins  
58 of  $\geq 10$  kDa are not strictly required for the bactericidal activity of EW at 45 °C<sup>14</sup>, despite  
59 potentially active. In addition, the  $\geq 10$  kDa proteins of EW were not required for lysis of *S.*  
60 *Enteritidis* in EW at 45 °C<sup>14</sup>. Thus, it was concluded that low mass (<10 kDa) components of  
61 EW (such as minerals and/or small bioactive/antimicrobial peptides) are probably the major  
62 contributors to the bactericidal activity of EW at 45 °C<sup>14</sup>.

63 The aim of the study presented here was to determine the key low-mass (<10 kDa) factors  
64 responsible for *Salmonella* killing by EW at 45 °C. To identify such factors, successive ultra-  
65 and nano-filtration steps were applied to EW (10 kDa, 1 kDa and 400 Da cut-off membranes,

66 respectively) and the antimicrobial activities and compositions of the resulting filtrates were  
67 determined.

## 68 **Materials and methods**

### 69 **Bacterial strain**

70 *Salmonella enterica* serovar Enteritidis NCTC13349 was kindly provided by Matthew  
71 McCusker (Center for Food Safety and Food Borne Zoonomics, Veterinary Sciences Centre,  
72 University College Dublin, Ireland). This strain was isolated from an outbreak of human food  
73 poisoning in the United Kingdom traced back to a poultry farm. The stock cultures were stored  
74 at -80 °C in 25% (v/v) glycerol. Before use, cells were propagated twice overnight at 37 °C in  
75 tryptic soy broth (TSB, Merck, Darmstadt, Germany) without shaking.

### 76 **Preparation of sterile egg white**

77 EW was prepared from 5 to 10 day-old eggs provided from a local supermarket. The eggshell  
78 surface was cleaned with a tissue, checked for cracks and then sterilized using 70% alcohol;  
79 residual alcohol was removed by briefly flaming the shell. Eggshells were then broken under  
80 sterile conditions and the egg whites were collected before aseptic homogenization with a DI25  
81 Basic homogenizer (Ika, Grosseron, Saint-Herblain, France) at 9,500 rpm for 1 min. The egg  
82 white pH was  $9.3 \pm 0.1$ .

### 83 **Egg-white fractionation**

84 EW ultrafiltration was carried out according to Baron *et al.*<sup>2</sup> using a pilot unit (Millipore type  
85 PRO LAB MSP 006239) equipped with an organic spiral-wound membrane (0.3 m<sup>2</sup>, 10 kDa  
86 cut-off). The concentrated EW (egg white retentate, EWR) was circulated back to the feed-tank  
87 while the EW filtrate (10kDa EWF) was drained off and collected in a beaker (Figure 1). The  
88 10kDa EWF was then either subjected to ultrafiltration (as above) using an organic spiral-

89 wound membrane (0.3 m<sup>2</sup>, 1 kDa cut-off) to obtain the 1kDa EWF, or to nanofiltration with a  
90 Helicon Nanomax 50 membrane (0.3 m<sup>2</sup>, 400 Da cut-off) to obtain the 400Da EWF (Figure 1).  
91 All EW filtrates (EWF) were sterilized by filtration (NalgeneR filter unit, pore size <0.2 μm,  
92 Osi, Elancourt, France), measured for pH and then stored at 4 °C until use.

### 93 **Physicochemical analyses.**

94 Nitrogen content of EW and EWFs was determined by the Kjeldahl method. Glucose was  
95 quantified using an enzymatic spectrophotometric test (Glucose GOD FS) according to the  
96 instructions of the provider (DiaSys GmbH, Germany). Mineral quantification by ICP-OES was  
97 carried out using samples in 10% iron-free nitric acid (Sigma-Aldrich; 438073), incubated in  
98 sealed, plastic tubes at 80 °C overnight with occasional vortexing. Samples were centrifuged (4  
99 °C, 30 min, 18,111g) and supernatants were diluted twofold. The multi-elemental contents of  
100 the nitric acid-dissolved sample-solutions were determined using a Perkin Elmer Optima 3000  
101 ICP-OES with radial view and a cross flow nebulizer (Anne Dudley, Analytical Technical  
102 Services, University of Reading).

### 103 **Mass spectrometry analysis**

104 Mass spectrometry (MS) analysis was performed on EW fractions (10kDa EWF, 400Da EWF  
105 and 400Da EWR) using a NanoLC Dionex U3000 system fitted to a Q-Exactive mass  
106 spectrometer (ThermoScientific, San Jose, USA) equipped with a nano-electrospray ion source.  
107 One hundred microliters of samples was diluted in a solution composed of 100 μL nano-LC  
108 solvent A described below and 50 μL of 2% formic acid. These samples were concentrated on  
109 a C18 PepMap100 cartridge (5 μm particle size, 100 Å pore size, 300 μm i.d., 5 mm length;  
110 Dionex, Amsterdam, The Netherlands), before peptide separation on a C18 PepMap100 column  
111 (3 μm particle size, 100 Å pore size, 75 μm i.d., 150 mm length; Dionex). Elution was  
112 performed using solvent A (2% v/v acetonitrile, 0.08% v/v formic acid and 0.01% v/v TFA in



113 deionized water) and solvent B (95% v/v acetonitrile, 0.08% v/v formic acid, and 0.01% v/v  
114 TFA in deionized water), by applying a gradient from 5 to 70% solvent B over 28 min followed  
115 by a gradient from 70 to 95% solvent B over 5 min at a flow rate of 0.3 mL/min.

116 Eluted peptides were directly electro-sprayed into the Proxeon source operating in positive ion  
117 mode with an optimized voltage of 2.1 kV. The mass spectra were recorded in a m/z range from  
118 250 to 2,000, with a resolution of the mass analyzer set to 70,000. For each scan, the ten most  
119 intense ions were selected for fragmentation. MS/MS spectra were recorded with a resolution  
120 set to 17,500, with exclusion from MS/MS fragmentation of the parent ion for 15 s. The  
121 equipment was externally calibrated according to the supplier's instructions. All samples were  
122 analysed in triplicate.

### 123 **Identification of peptides**

124 Peptides were identified from the MS/MS spectra using X!Tandem pipeline software  
125 (Plateforme d'Analyse Protéomique de Paris Sud-Ouest (PAPPSO), INRAE, Jouy-en-Josas,  
126 France, <http://pappso.inra.fr>). The search was performed against a database composed of  
127 reviewed proteins of *Gallus gallus* (2262 proteins downloaded to which was added the common  
128 Repository of adventitious Protein, <http://thegpm.org/crap>). Database search parameters were  
129 specified as follows: non-specific enzyme cleavage; a 0.05 Da mass error for fragment ions;  
130 10 ppm mass error for parent ions; with methionine oxidation and serine phosphorylation as  
131 putative modifications. A minimum score corresponding to an e-value below 0.05 was required  
132 for valid peptide identification.

### 133 **Prediction of antimicrobial activity of peptides**

134 According to an approach previously described by Bishop *et al.*<sup>15</sup>, the peptide sequences  
135 identified in the EW fractions were submitted to the free web-based ADAM database<sup>16</sup> using

136 SVM                      Predict                      (Support                      Vector                      Machine)

137 ([http://bioinformatics.cs.ntou.edu.tw/ADAM/svm\\_predict.php](http://bioinformatics.cs.ntou.edu.tw/ADAM/svm_predict.php)) or to the cAMP database using  
138 SVM, Random Forest (RF), Artificial Neural Network (ANN) and Discriminant Analysis (DA)  
139 (<http://www.camp.bicnirrh.res.in/predict/hii.php>). Several physicochemical characteristics of  
140 these peptides were also calculated using ProtParam tools (ExPASy Bioinformatics Resource  
141 Portal): theoretical molecular weight, theoretical pI, hydrophobicity evaluated by the GRAVY  
142 index (Grand Average Hydropathy value) and stability evaluated by the Instability index. The  
143 net charge at pH 7.0 and pH 9.0 was predicted using the Protein Calculator v3.4  
144 (<https://protcalc.sourceforge.net/cgi-bin/protcalc>). Comments about structure features were  
145 extracted from the Antimicrobial Peptide Calculator and Predictor APD3  
146 (<http://aps.unmc.edu/AP/prediction/actionInput.php>).

#### 147 **Peptide synthesis**

148 The peptides P1=FVPPVQR, P2=GDPSAWSWGAEAHS, P3=TPPFGGFR, and  
149 P4=HPFIQHPVHG were synthesized by Eurogentec (Angers, France) at purity rates above  
150 95%. Stock solutions were prepared by dissolving each synthetic peptide in sterile ultrapure  
151 water at 2 mg/mL and stored at -20 °C until use.

#### 152 **Anti-*Salmonella* activity measurement**

153 The anti-*Salmonella* activity of EW, EWFs and isolated EW peptides was determined by  
154 incubation with *Salmonella* for 24 h at 45 and 30 °C (as a control temperature), as follows.  
155 After overnight propagation in tryptone soy broth (TSB, pH 7.3, Merck, Darmstadt, Germany),  
156 *Salmonella* cultures were centrifuged (5,600g at 15 °C for 7 min) and cells were washed three  
157 times in the same volume of tryptone salt medium (AES, Combourg, France) or TSB (when  
158 TSB was used as the assay medium). The washed pellets were finally resuspended in the same  
159 volume of tryptone salt medium and diluted to inoculate at 2% 96-well microplates 2.2 mL

160 (Starlab, Bagneux, France) containing 800  $\mu$ L of the assay medium to obtain a final *Salmonella*  
161 inoculum level of  $6\pm 0.2 \log_{10}$  CFU/mL.

162 To test the antibacterial activity of the peptides of interest, assay medium with synthetic P1, P2,  
163 P3 or P4 peptides (100  $\mu$ g/mL) in either 400Da EWF or minimal medium M63 (KH<sub>2</sub>PO<sub>4</sub> 60  
164 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.5 mM, MgSO<sub>4</sub> 1 mM, glucose 0.4%) were used; pH was adjusted to 9.2 with  
165 KOH 30%.

166 To test the effect of pH and nutritional deficiency, the pH of the 400Da EWF was adjusted to  
167 9.2 with 2 M NaOH, and glucose and NH<sub>4</sub>Cl were added to a final concentration of 25 and  
168 3mM, respectively.

169 After incubation for 24h at 30°C, viable cell numbers were determined using a numeration  
170 method based on the miniaturization of the conventional plate-counting technique, according  
171 to Baron *et al.*<sup>17</sup> with a Tryptone soya agar (TSA) (Merck, Darmstadt, Germany) overlay  
172 procedure. Results were compared using analysis of variance and the average comparison test  
173 using the R 2.13.0 software (<http://cran.r-project.org>).

## 174 **Results and Discussion**

### 175 **Removal of components >400 Da significantly reduces, but does not eliminate the** 176 **bactericidal activity of EW against *S. Enteritidis* at 45°C**

177 The approach adopted to investigate the key factors responsible for the bactericidal effect of  
178 EW and EWFs on *S. Enteritidis* at 45 °C was based on an EW fractionation strategy using  
179 successive ultrafiltration and nanofiltration steps (10 kDa, 1 kDa and 400 Da cut-off,  
180 respectively) followed by assessment of *S. Enteritidis* survival at 45 °C (and 30 °C as a control)  
181 in EW and in the three resulting fractions: 10kDa EWF, 1kDa EWF, and 400DaEWF.

182 A strong bactericidal effect was observed after 24 h at 45 °C in all EW fractions: *Salmonella*  
183 cells were undetectable in EW, 10kDa EWF and 1kDa EWF which corresponds to a 6  $\log_{10}$   
184 reduction in cell numbers. However, *S. Enteritidis* only decreased by  $2.6 \pm 0.5 \log_{10}$  in the 400Da

185 EWF (Figure 2A). The bactericidal effect observed was not simply due to temperature as there  
186 was an increase of  $2.6 \pm 0.2 \log_{10}$  CFU/mL after 24 h when incubation at 45 °C was performed  
187 in TSB rather than EW or the EWFs. However, the bactericidal effect was only observed for  
188 EW and EWFs at 45 °C; at 30 °C the *Salmonella* cells count increased in all the media tested  
189 (Figure 2B). Nevertheless, the growth at 30 °C was significantly lower in EW and in EWFs  
190 ( $+1.5 \pm 0.7 \log_{10}$  CFU/mL in EW;  $+2.4 \pm 0.2 \log_{10}$  CFU/mL in 10kDa and 1kDa EWF, and  
191  $+1.1 \pm 0.2 \log_{10}$  CFU/mL in 400Da EWF) than in TSB medium ( $+3.4 \pm 0.1 \log_{10}$  CFU/mL). The  
192 above results are in agreement with those previously obtained in EW and 10kDa EWF at 30 °C<sup>2</sup>  
193 and 45 °C<sup>10,14</sup>, and they indicate that EW and the EWFs allow significant growth of *S.*  
194 *Enteritidis* at 30 °C, but become strongly bactericidal at 45 °C, unlike standard growth medium.  
195 Importantly, the bactericidal activity towards *S. Enteritidis* at 45 °C was significantly reduced  
196 for the 400Da EWF suggesting that EW factors larger than 400 Da play a major role in the  
197 bactericidal activity of EW at this temperature.

198 In order to confirm the differences in the bactericidal activity of the 400Da EWF and the other  
199 EW fractions, *S. Enteritidis* survival was measured at 45 °C (and 30 °C as a control) in the  
200 400Da EWF with addition of the 400Da EWR at 0-100% (v/v) concentration (Figure 3). A clear  
201 dose-dependent response was observed, with a progressive increase in bactericidal activity at  
202 45 °C achieved as the percentage of 400Da EWR was elevated, with the activity reaching a  
203 maximum 6  $\log_{10}$  reduction with 100% (v/v) 400Da EWR (Figure 3A), as was obtained for the  
204 1kDa and 10kDa EWFs (Figure 2A). Addition of the 400Da EWR to the 400Da EWF also  
205 restored the growth of *S. Enteritidis* at 30 °C (Figure 3B), such that the same level of growth  
206 was seen as that obtained for the 1kDa or 10kDa EWFs (Figure 2B). This indicates that the  
207 400Da EWR provides a source of nutrients for *S. Enteritidis* growth at 30 °C, but contributes to  
208 the antibacterial activity observed at 45 °C. In summary, the above data indicate that the  
209 bactericidal components of EW can be separated into two fractions on the basis of mass (> and

210 <400 Da), and that recombining these fractions restores the bactericidal activity obtained at  
211 45 °C to match that seen for whole EW. The results therefore supports that the bactericidal  
212 activity of EW at 45 °C is a multifactorial phenomenon<sup>1</sup>, and suggests it may result from the  
213 combination of physicochemical factors, and small molecules (< 10 kDa and >400 Da) such as  
214 antimicrobial peptides.

#### 215 **Contribution of physicochemical factors to the bactericidal activity of EW at 45 °C**

216 Chemical analysis of EW, and the 10kDa and 400Da EWFs was performed (Table 1) to  
217 determine whether there are any differences that could explain the reduced bactericidal activity  
218 seen for the 400Da EWF. The glucose (180 Da) concentration in EW and 10kDa EWF (21 and  
219 25 mM, respectively) was approximately twofold higher than that typically used in culture  
220 media (around 11 mM glucose); a similar glucose concentration (17 mM) was also measured  
221 in 1kDa EWF (data not shown). This suggests that there is sufficient glucose in EW but also in  
222 10kDa EWF and 1kDa EWF to support *S. Enteritidis* growth. However, it is possible that the 4  
223 to 5-fold lower level of glucose (4.8 mM) in the 400Da EWF might contribute to the lower  
224 growth observed at 30 °C in 400Da EWF in comparison to 10kDa EWF (+1.1±0.2 log<sub>10</sub>  
225 CFU/mL and +2.4±0.2 log<sub>10</sub> CFU/mL, respectively). However, it is unlikely that this  
226 difference in glucose content is responsible for the reduced bactericidal activity of the 400Da  
227 EWF at 45 °C.

228 The total nitrogen concentration was much higher in EW (1364 mM) than in the 10kDa and  
229 400Da EWFs (2.3 and 0.69 mM, respectively), which is consistent with the high level of protein  
230 (around 10% w/v) in EW and the loss of protein from the EWFs through filtration (Table 1);  
231 the nitrogen content in 1kDa EWF (2.1 mM) was similar to that measured in 10kDa EWF (data  
232 not shown). The low nitrogen concentration of 400Da EWF is close to the threshold  
233 concentration (1 mM) for enterobacteria growth<sup>18</sup>. As for glucose, this relatively low nitrogen

234 availability could contribute to the lower growth of *S. Enteritidis* at 30 °C in the 400Da EWF  
235 compared to 10kDa EWF, as suggested by Figure 4. However, it is unlikely that this low protein  
236 content is responsible for the reduced bactericidal activity of 400Da EWF at 45 °C. As for the  
237 lower growth at 30°C in EW in comparison to 10kDa EWF and 1kDa EWF, it was likely due  
238 to the presence of antimicrobial proteins in EW<sup>4</sup>.

239 For the eight major minerals presented in Table 1, some differences were found between EW  
240 and the EWFs. In particular, there were major decreases in iron and manganese, and modest  
241 decreases for zinc, copper, calcium, potassium and magnesium in the EWFs compared to EW  
242 (7.5-900, 250-500, 5-36, 3-9, 5-12, 0.98-1.25, 1.11-4 fold, respectively; Table 1). However,  
243 except for iron, the measured mineral concentrations are above the concentration thresholds  
244 considered necessary for bacterial growth<sup>19-24</sup>. EW is well recognized as an iron-deficient  
245 medium and it is generally considered that in whole EW iron is almost entirely bound to  
246 ovotransferrin<sup>4</sup> which would be lost upon filtration; this explains why the 10kDa and 400Da  
247 EWFs (both ovotransferrin-free) contain up to 900-fold less iron than EW. The reduced Zn, Cu  
248 and (particularly) Mn in the EWFs suggest that these metals are also retained; this is likely to  
249 be due to association with EW macromolecules<sup>25</sup>. However, such reductions in mineral levels  
250 are unlikely to explain the reduced bactericidal activity of the 400Da EWF toward *S. Enteritidis*  
251 at 45 °C (or reduced growth seen in 400Da EWF at 30 °C) since levels of these minerals are  
252 similar in the 10kDa and 400Da EWFs (Table 1).

253 One last difference between the 400Da EWF, and EW and the 10kDa EWF, is pH which was  
254 lower in the 400Da EWF (Table 1). The importance of alkaline pH for the antimicrobial activity  
255 is well reported<sup>1,6</sup>. Therefore, it is reasonable to suggest that the lower pH measured in the  
256 400Da EWF could partly explain the lower bactericidal activity of this fraction compared to  
257 EW and 10kDa EWF. From all the physicochemical characteristics of 400Da EWF determined  
258 here, its pH (8.7) is the most likely hypothesis to explain the least bacteria destruction observed

259 at 45 °C in this medium in comparison with 10kDa EWF. To test whether the reduced pH,  
260 glucose or nitrogen concentrations of the 400Da EWF compared to EW and 10kDa EWF could  
261 account for its reduced bactericidal activity, *Salmonella* survival was measured in the 400Da  
262 EWF at 45 °C (and 30 °C as control) at pH 8.7 and 9.2, with glucose at 25 mM and nitrogen at  
263 3 mM (final concentrations) (Figure 4). The modifications of 400Da EWF did not significantly  
264 change ( $p>0.05$ ) bactericidal activity at 45 °C (Figure 4A), indicating that the changes in pH,  
265 glucose and nitrogen availability are not responsible for the reduced bactericidal activity of the  
266 400Da EWF compared to the 10kDa EWF. However, the combined increase in pH, nitrogen  
267 and glucose content of the 400Da EWF did result in a significant increase in *S. Enteritidis*  
268 growth at 30 °C, although growth was still lower than that obtained in 10kDa EWF (Figure 4B).  
269 These findings indicate that nutrient (carbon and/or nitrogen sources) availability and pH are  
270 factors that impact *S. Enteritidis* growth in EW at 30 °C. Since adjusting pH, glucose and  
271 nitrogen availability only partly restored growth in 400Da EWF at 30 °C towards that seen in  
272 the 10kDa EWF, it is likely that there are other differences between these filtrates that affect  
273 growth. As the iron, zinc, copper and manganese levels are similar in the 400Da EWF and the  
274 10kDa EWF, it is unlikely that differences in availability of these metals would explain the  
275 difference in growth. So other factors are likely responsible for this effect.

#### 276 **Putative antimicrobial peptides (AMPs) are present in EW ultrafiltrates**

277 To test the possible involvement of small bioactive compounds in the bactericidal activity of  
278 EW and EWFs at 45 °C, the 10kDa and 400Da EWFs, and 400Da EWR were analysed by mass  
279 spectrometry (MS). No peptides could be detected in the 400Da EWF, consistently with the  
280 very low nitrogen content measured in this fraction (equivalent to a peptide content of 0.06 g/L)  
281 but 12 peptides were identified in the 10kDa EWF, the peptide content of which was estimated  
282 at 0.2 g/L. All 12 peptides were also detected in the 400Da EWR in addition with 10 other  
283 peptides (Table 2). The higher number of peptides detected in the 400Da EWR likely results

284 from a higher concentration in the retentate (peptide content estimated at 0.44 g/L) with respect  
285 to that in the more diluted 10kDa EWF. However, due to the detection threshold of LC-MS/MS  
286 analysis, it is likely that other peptides present at very low concentration might exist in EWFs.  
287 Similarly, because of technical limits which make impossible the identification of peptides  
288 smaller than 5 to 6 amino acid residues and those larger than 40 to 45 amino acid residues, the  
289 list of peptides detected in 10kDa EWF and 400Da EWR is likely not exhaustive. In particular,  
290 it is noteworthy that avian beta-defensin 11 (AvBD11; 82 amino acid residues), gallin  
291 (OvoDA1; 41 amino acid residues) and OvoDB1 (45 amino acid residues), all previously  
292 identified in EW, were not detected in the present study<sup>4,26,27</sup>.

293 The peptides identified originate mainly from ovocleidin-116 and clusterin, two minor proteins  
294 previously identified in EW<sup>28,29</sup> (Table 2). Ovocleidin-116 is a major component of the eggshell  
295 matrix, and a main actor of the regulation of eggshell calcification<sup>30</sup>. Hen egg clusterin is a  
296 structural component of the eggshell matrix, but also identified in EW<sup>31</sup>; clusterins are  
297 ubiquitous proteins with molecular chaperone function<sup>32</sup>. Among the 11 peptides stemming  
298 from clusterin, four belong to the fragment [203-221: TPPFGGFREAFVPPVQRVR] (group 3,  
299 Table 2), five to the fragment [211-221: EAFVPPVQRVR] (group 1, Table 2), and two to the  
300 fragment [232-246: EIHPFIQHPVHGFHR] (group 4, Table 2). Among the nine peptides  
301 derived from ovocleidin-116 (group 5, Table 2), two belong to the fragment [459-482:  
302 VQQEVAPARGVVGGMVVPEGHRAR], six to the fragment [561-587:  
303 IGQAARPEVAPAPSTGGRIVAPGGHRA], and one corresponds to the fragment [622-643:  
304 STDVPRDPWVWGS AHPQAQHTR]). Moreover, two peptides originate from zona pellucida  
305 sperm binding protein 3, called ZP3. ZP3 is one of the five ZPs present in the vitelline  
306 membrane of bird eggs, all playing an important role in egg fertilization. ZP3 is especially  
307 involved in the binding of sperm in the germinal disc region of the yolk<sup>33,34</sup>. Both peptides  
308 stemming from this protein and identified in 400Da EWR belong to the fragment [86-99:



309 GDPSAWSWGAEAH[S] (group 2, Table 2). To the best of our knowledge, no antibacterial  
310 activity has been ever reported for ovocleidin-116, hen egg clusterin, and ZP3.

311 The main physicochemical properties of the peptides are summarized in Table 2. Their  
312 molecular weight ranges from 722 to 2,528 Da, and their predicted pI from 3.39 to 11.8. A high  
313 proportion of these peptides (16 out of 22) are likely to form an  $\alpha$ -helix. Moreover, most (17  
314 out of 22) are predicted to be positively charged at neutral pH, and 13 are predicted to remain  
315 positively charged at pH 9 (close to the pH of 9.3 used in the present study, that is the natural  
316 EW pH a few days after laying). A positive net charge and helicity are well known  
317 characteristics of AMPs<sup>35</sup>. To further probe the potential antibacterial activity of the peptides  
318 identified, a bioinformatics approach was applied.

319 All the peptide sequences identified were evaluated for their potential antimicrobial activity  
320 using web-based prediction tools in the ADAM and cAMP-databases (see M&M section). Nine  
321 peptides presented a negative ADAM score and were not considered for further analysis. All  
322 nine of these peptides stemmed from ovocleidin-116 (group 5, Table 2). In contrast, 13 peptides  
323 achieved a positive ADAM score ranging from 0.61 to 2.31. These 13 peptides can be divided  
324 into four groups, based on the shortest common sequence (Table 2). As a complement to this  
325 analysis based on the ADAM database, the cAMP prediction scores were calculated for these  
326 13 peptides, using four different algorithms. To enable experimental determination of the  
327 antimicrobial activity of representative peptides from the set identified, four peptides were  
328 selected for synthesis on the basis of the following criteria: i) the peptide showing the highest  
329 ADAM score within each of the 4 groups (1-4) of relevance (Table 1); and ii) possessing at  
330 least one positive cAMP database score. Thus, four peptides (designated P1, P2, P3 and P4 in  
331 Table 2) were selected.

332 With a GRAVY index score above zero, P3 is considered a hydrophobic peptide, whereas P2  
333 and P4 are mostly hydrophilic; P1 has a predicted intermediary hydrophobic/hydrophilic nature

334 (Table 2). Moreover, out of the four potential AMPs selected, P2 is the only one likely to form  
335 a  $\alpha$ -helix, whereas P1 and P3 are rich in proline residues, well-known for their “helix-breaker”  
336 effect<sup>36</sup>. P1 and P3 are also predicted to be structurally unstable, based on Instability Index,  
337 whereas P2 and P4 are predicted as stable (Table 2).

338 **Two out of the four putative EW AMPs selected exert a bactericidal activity against *S.***  
339 ***Enteritidis* at 45 °C**

340 To experimentally determine the antimicrobial activity of the four selected predicted AMPs, *S.*  
341 *Enteritidis* survival was assessed at 45 °C (and 30 °C for control) in 400Da EWF with  
342 chemically synthesized P1, P2, P3 or P4 peptides, and the bacterial enumeration was compared  
343 to that obtained in 10kDa EWF, 400Da EWF and TSB (Figure 5).

344 None of the four peptides tested displayed antibacterial activity at 30 °C (Figure 5B).  
345 Additionally, the P3 and P4 peptides had no effect on the bactericidal activity of 400Da EWF  
346 at 45 °C (Figure 5A). In contrast, the P1 and P2 peptides (at 100  $\mu$ g/mL; 119 and 69  $\mu$ M for P1  
347 and P2, respectively) strongly increased ( $p < 0.001$ ) the bactericidal activity of 400Da EWF at  
348 45 °C. Indeed, the addition of either P1 or P2 resulted in a substantial 6 log<sub>10</sub> reduction of *S.*  
349 *Enteritidis* that is the same bactericidal effect than that observed for 10kDa EWF at 45 °C  
350 (Figure 5A). Therefore, the results suggest that P1 and P2 contribute to the bactericidal activity  
351 of EW and EW ultrafiltrates at 45 °C. The effect of concentration on the bactericidal activities  
352 of P1 and P2 was also tested, and the results show a dose-dependent response for both peptides  
353 at 45 °C in 400Da EWF over a concentration range from 0 to 100  $\mu$ g/mL, with a higher  
354 bactericidal effect for P2 (Figure 6B) than for P1 (Figure 6A).

355 Thus, the P1 and P2 peptides can be classified as bactericidal peptides active against *S.*  
356 *Enteritidis* under the specific conditions of EW or EW ultrafiltrates at 45 °C. Since 45 °C is  
357 close to the body temperature of the hen, P1 and P2 are likely to play a role in resisting

358 *S. Enteritidis* infection during egg formation. However, the P1 and P2 peptides displayed no  
359 bactericidal activity in M63 minimal medium, even at 45 °C, either at pH 7.8 or 9.2 (Figure 7).  
360 Then, P1 and P2 peptides cannot explain by themselves the bactericidal activity of EW and EW  
361 ultrafiltrates at 45 °C. Actually, it is very likely that both peptides interact in EW, as well as in  
362 10kDa and 1kDa EWFs, with other harmful factors such as nutrient deprivation, alkaline pH,  
363 or other unknown antimicrobial compounds.

364 To test any synergistic action for the P1 and P2 peptides, the bactericidal effect of combining  
365 the two peptides in 400Da EWF at 45 °C was examined (Figure 8). The results show a clear  
366 synergistic effect for a 1:1 w/w combination of P1 and P2 (25 µg/mL total concentration) with  
367 a higher bactericidal activity compared to that obtained for each peptide alone at the same  
368 concentration (Figure 8). A 6 log<sub>10</sub> reduction of *S. Enteritidis* was obtained after 24 h incubation  
369 with the peptide mixture, whereas only 2.55±0.48 and 4.5±0.15 log<sub>10</sub> reductions were obtained  
370 with P1 and P2 alone, respectively (Figure 8). Combining P1 and P2 had no apparent effect on  
371 *S. Enteritidis* growth in 400Da EWF at 30 °C compared to that observed in the absence of  
372 peptides (data not shown).

373 To conclude, this study has advanced understanding of the bactericidal activity of EW at 45 °C.  
374 In particular, two new AMPs (P1 and P2) have been identified in EW and their likely  
375 involvement in the bactericidal activity of EW has been revealed. The P1 and P2 peptides have  
376 characteristics commonly attributed to AMPs. These characteristics include a total hydrophobic  
377 ratio (defined using the APD tool: <http://aps.unmc.edu/AP/><sup>37</sup>) of 42% and 35% for P1 and P2,  
378 respectively, which matches the relatively high proportion (≥30% or more) of hydrophobic  
379 residues often associated with AMPs<sup>35</sup>. Moreover, P1 contains two Pro residues (28% of all  
380 residues) and one Arg residue (14%), whereas P2 contains two Trp (14%), one Pro (7%) and  
381 one His (7%) residues, which are common features of AMPs<sup>35,37</sup>. Furthermore, according to the  
382 APD tool for structure prediction, P2 may form a α-helix with at least three residues on the

383 same hydrophobic surface, suggesting an amphiphilic helix folding pattern, as hypothesized for  
384 AMPs such as magainins or cecropins; this property is thought to promote interaction with the  
385 bacterial membrane<sup>35,38</sup>. Lastly, P1 has 43% similarity to an AMP registered in the APD  
386 database under ID AP02431 (TPPQS), which originates from *Bacillus subtilis*<sup>39</sup>, while P2 has  
387 43% similarity to another AMP registered under ID AP02938 (GTAWRWHYRARS), obtained  
388 from the rumen microbiome<sup>40</sup>. P1 has a predicted alkaline pI (pI=9.75) and thus would be very  
389 slightly cationic at pH 9 (i.e. close to the pH here tested), while P2 is an acidic peptide (pI=4.35).  
390 Thus, under the conditions tested here, neither P1 nor P2 have the strong cationic characteristics  
391 widely reported for AMPs, and regarded as critical for interaction between AMPs and bacterial  
392 membranes, which is considered to be the first step leading to AMP-mediated membrane  
393 dysfunction and disruption<sup>35</sup>. Nonetheless, some anionic or non-cationic peptides are proven  
394 AMPs<sup>38</sup>, suggesting that a cationic characteristic is not a strict requirement for AMP  
395 functionality. In any case, it is likely that P1 and P2 do not act like typical AMPs, since their  
396 most striking feature is that their activity requires both a permissible temperature (45 °C) and a  
397 specific medium composition (EW or EWF).

398 Despite the original features of P1 and P2 in comparison to most of AMPs, the assumption of  
399 membrane disruption induced by these peptides is preferred. Indeed, a previous study evidenced  
400 membrane damage (inner and outer membranes) on *E. coli* during incubation in same  
401 conditions, *i.e.* in EW at 45°C<sup>41</sup>. Moreover, the influence of temperature on P1 and P2  
402 bactericidal activity could be related to membrane fluidity as high temperatures increase the  
403 fluidization of biological membranes<sup>42</sup>. Then, the ability of antimicrobial components to cross  
404 and/or disrupt the bacterial membrane increases as membrane fluidity rises. The mechanism  
405 governing the observed synergy between P1 and P2 is unclear, but this finding highlights the  
406 potential for synergistic action of antimicrobial components in EW.

407 Finally, this study confirms the antibacterial role of the EW peptide fraction, besides that of  
408 antibacterial proteins described for a long time<sup>4</sup>. It is especially significant as few is known  
409 about the antibacterial peptides naturally present in EW. Despite a great number of peptides  
410 have been identified in EW during the last decades thanks to proteomics, the biological  
411 functions of most of them, and especially their antimicrobial activities have still to be  
412 investigated<sup>4</sup>. To date, an avian- $\beta$ -defensin and a gallin have been identified in hen EW<sup>43</sup> and  
413 their antibacterial activities have been confirmed<sup>26,44</sup>. These natural peptides both belong to the  
414 family of defensins which are part of the innate immune system in many living species. Avian-  
415  $\beta$ -defensins are cationic peptides of 1 to 9 kDa identified in the eggs of several bird species<sup>45</sup>.  
416 These peptides are expressed in many different tissues, including the hen oviduct<sup>46</sup>, which  
417 explains that the different compartments of hen egg contain avian- $\beta$ -defensins which are  
418 supposed to be involved in the protection of the embryo during hatching<sup>26</sup>. Ovodefensins, a  
419 sub-family of  $\beta$ -defensins including gallin (4732 Da), have been also identified in the EW of  
420 different bird species<sup>4</sup>. Moreover, it is more than likely that EW contain many other  
421 antimicrobial peptides, not yet identified, as indicated by the consequence of EW treatment  
422 with proteinase K. This treatment eradicated the anti-Salmonella activity of a 3kDa EWF,  
423 suggesting that antimicrobial polypeptides smaller than 3 kDa play an active role in the  
424 antibacterial defence of EW<sup>47</sup>. However, what does differ between both peptides identified in  
425 the present study and antimicrobial peptides such as defensins, is that P1 and P2 are not  
426 expressed as such from the hen genome, but are stemming from larger proteins, namely  
427 clusterin and ZP3, respectively. This consequently indicate that these proteins have been  
428 hydrolysed *in situ*. It is noteworthy that in quail eggs, a 26 amino acid sequence containing a  
429 homologous sequence of P2 peptide was removed from ZP3 after ovulation, presumably by a  
430 protease secreted in the infundibulum<sup>48</sup>. This might explain why P2 peptide which stems from  
431 a vitelline membrane protein (ZP3) was found in EW. It could be hypothesized that this peptide,

432 released from the vitelline membrane into the forming EW after ovulation, could play a role in  
433 protecting the embryo during the completion of egg formation in the oviduct. The fact that P2  
434 peptide specifically acts at 45°C, close to the hen body temperature, supports this assumption.  
435 More generally speaking, protein degradation during formation and/or storage of eggs was  
436 previously reported, based on the decrease of the band intensity of some proteins in  
437 electrophoresis<sup>49</sup>, and more recently, the release of small peptides (<10 kDa and <3kDa) was  
438 also established<sup>50</sup>. However, the mechanisms responsible for the proteolysis still remain  
439 unknown in most cases. Various proteases naturally present in EW<sup>51</sup> could catalyse the  
440 proteolysis. Self-degradation of proteins has been also described as a spontaneous and quite  
441 universal phenomenon<sup>52</sup>. However, only small peptides stemming from ovotransferrin,  
442 ovomucin, ovomucoid and ovoinhibitor, i.e. major proteins, have been described in EW to  
443 date<sup>50</sup>. In the present study, it is noteworthy that the EW fractionation strategy using ultra- and  
444 nanofiltration membranes, leading to a concentrated fraction (400Da EWR), enabled the access  
445 to peptides stemming from minor EW proteins. Then, whereas protein degradation can be seen  
446 as a potentially detrimental phenomenon when it concerns antimicrobial proteins  
447 (ovotransferrin, lysozyme, ovoinhibitor, ovomucoid), the present study highlights it could also  
448 contribute to a higher protection of eggs against bacteria, thanks to the release of antimicrobial  
449 peptides from non-antimicrobial proteins such as clusterins and ZP3. Beyond the specific issue  
450 of egg protection, this study also underlines that egg white proteins, even non-antimicrobial  
451 ones, should be considered as potential natural sources of antimicrobial peptides. This has  
452 special relevance where innovative antimicrobial molecules are being sought to counteract  
453 increasing bacterial resistance which is a major public health challenge.

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#### 465 **Abbreviations used**

466 EW, Egg White; EWR, Egg White Retentate; EWF, Egg White Filtrate; AMP, Antimicrobial  
467 Peptide; cAMP, Collection of Antimicrobial Peptides; SVM, Support Vector Machine; RF,  
468 Random Forest; ANN, Artificial Neural Network; DA, Discriminant Analysis; GRAVY, Grand  
469 Average Hydropathy Value; APD, Antimicrobial Peptide Database; Nano LC-MS/MS;  
470 Nanoscale Liquid Chromatography coupled to tandem mass spectrometry; ICP-OES,  
471 Inductively Coupled Plasma-Optical Emission Spectrometry.

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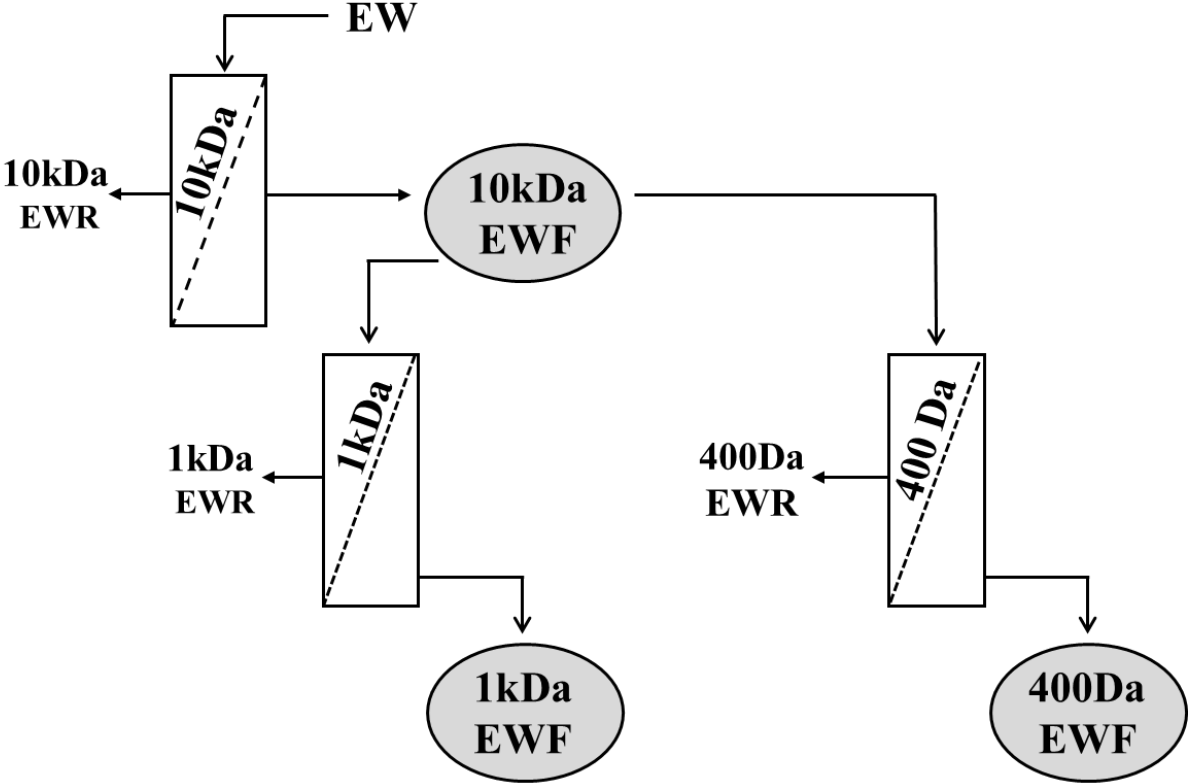
**Table 1:** Main physicochemical characteristics of 10kDa and 400Da egg white filtrates (EWF) and of egg white (EW) (\*data from literature<sup>53-57</sup>).

	<b>EW</b>	<b>10kDa EWF</b>	<b>400Da EWF</b>
<b>pH</b>	9.3	9.2	8.7
<b>glucose (mM)</b>	25	21.5	4.8
<b>Total N (mM)</b>	1364	2.3	0.69
<b>Na (mM)</b>	67.4 to 80.9*	96.1	62.7
<b>K(mM)</b>	35.8 to 44.2*	44.7	28.7
<b>Ca (mM)</b>	1.2 to 2.9*	0.96	0.24
<b>Iron (mM)</b>	0.003 to 0.018*	<0.00002	0.00044
<b>Mg (mM)</b>	3.7 to 4.9*	3.32	0.92
<b>Zn (mM)</b>	0.005 to 0.018*	0.0005	0.001
<b>Cu (mM)</b>	0.003 to 0.006*	0.00098	0.00072
<b>Mn (mM)</b>	0.001 to 0.002*	<4.18e-06	<4.18e-06

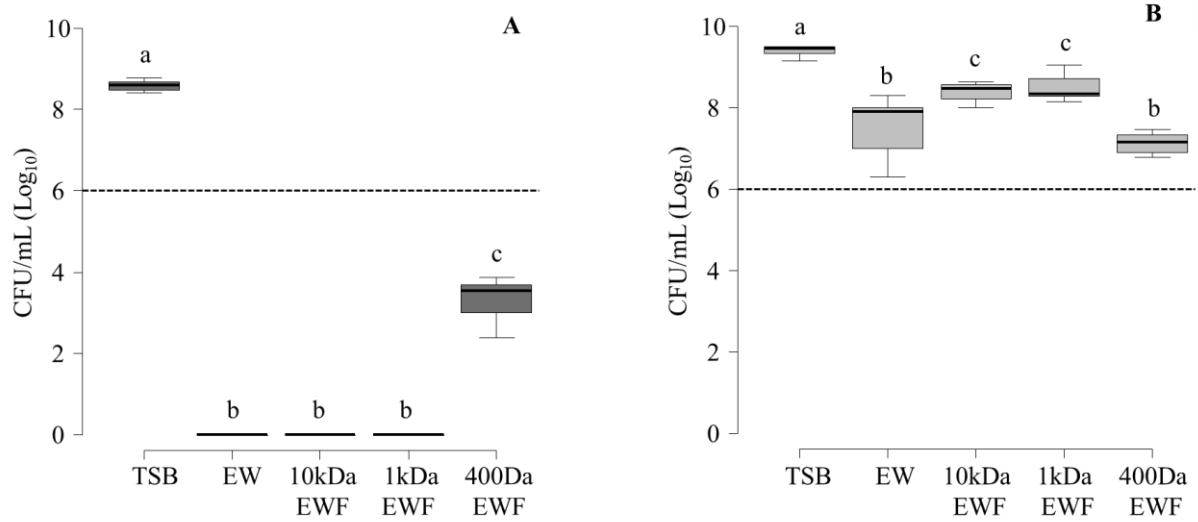
**Table 2.** Sequences of the peptides identified in 10kDa EWF, 400Da EWR and 400Da EWF, divided in five groups based on the shortest common sequence. Antimicrobial property was predicted from the ADAM database using SVM (Support Vector Machine), or from the cAMP database using SVM, RF (Random Forest), ANN (Artificial Neural network) or DA (Discriminant Analysis); for each prediction method, peptides are regarded as antimicrobial (AMP) or not antimicrobial (NAMP). “Origin” indicates the protein from which each peptide originates, and “Fragment” indicates the positions of the first and last amino acid residues in the protein sequence. Physicochemical properties are either experimental (MW, molecular weight determined by mass spectrometry) or theoretical, predicted using ProtParam tools (MW; pI, isoelectric point; GRAVY, hydrophobicity index; Instability index), or Protein Calculator v3.4 (net charge at pH 7.0 and 9.0). Structure features predicted using APD3 (Antimicrobial Peptide Calculator and Predictor) are indicated as “Comments”. Peptides selected for further study (P1-P4) are indicated in bold.

GROUPS	PEPTIDES	presence in		ADAM Score	cAMP Prediction Score					ORIGIN	Physicochemical characteristics							
		10kDa EWF	400Da EWF		SVM	RF	ANN	DA	Fragment		Experimental MW	Theoretical MW	pI	Net charge at pH7	Net charge at pH9	GRAVY	Instability Index	Comments
		X	X		-	-	-	-	-									
1	A F V P P V Q R	X	X	-	AMP 1.66	NAMP 0.057	NAMP 0.354	AMP	NAMP 0.020	212-219	chlaesrin precursor (NP_990231.1)	913.09	913.52	9.79	0.9	0.1	0.225	rich in P
	A F V P P V Q R V	-	X	-	AMP 1.46	NAMP 0.139	NAMP 0.450	AMP	NAMP 0.064	212-220		1012.22	1012.59	9.79	0.9	0.1	0.667	putative leaky
	E A F V P P V Q R	X	X	-	AMP 0.99	NAMP 0.008	NAMP 0.277	NAMP	NAMP 0.001	211-219		1042.20	1042.57	6.10	-0.1	-0.9	-0.189	putative leaky
	E A F V P P V Q R V R	X	X	-	AMP 0.65	NAMP 0.013	NAMP 0.165	NAMP	NAMP 0.080	211-221		1297.53	1297.74	9.70	0.9	0.1	-0.182	putative leaky
2	F V P P V Q R R = P1	X	X	-	AMP 2.07	NAMP 0.000	NAMP 0.363	AMP	NAMP 0.012	213-219	842.01	842.49	9.75	0.9	0.1	0.000	rich in P	
	D P S A W S W G A E A H S	-	X	-	AMP 0.61	NAMP 0.075	NAMP 0.118	NAMP	NAMP 0.018	87-99	1400.43	1400.59	4.35	-1.8	-2.9	-0.846	putative leaky	
	G D P S A W S W G A E A H S = P2	-	X	-	AMP 0.90	NAMP 0.015	NAMP 0.170	NAMP	NAMP 0.108	86-99	1457.48	1457.61	4.35	-1.8	-2.9	-0.814	putative leaky	
	T P P F G G F = P3	X	X	-	AMP 2.25	AMP 0.999	NAMP 0.432	NAMP	NAMP 0.143	203-209	721.81	722.35	5.19	-0.1	-0.9	0.129	rich in P or G	
3	T P P F G G F R	X	X	-	AMP 1.96	AMP 0.690	NAMP 0.338	AMP	NAMP 0.177	203-210	878.00	878.45	9.41	0.9	0.1	-0.450	rich in P or G	
	T P P F G G F R E A F V P P V Q R V	-	X	-	AMP 1.39	NAMP 0.055	NAMP 0.139	NAMP	NAMP 0.114	203-220	2001.32	2001.07	9.26	0.9	0.1	-0.061	putative leaky	
	T P P F G G F R E A F V P P V Q R V R	-	X	-	AMP 1.29	NAMP 0.049	NAMP 0.259	NAMP	NAMP 0.178	203-221	2157.51	2157.17	11.70	1.9	1.1	-0.295	putative leaky	
	H P F I Q H P V H G = P4	-	X	-	AMP 2.31	NAMP 0.390	NAMP 0.240	NAMP	NAMP 0.010	234-243	1168.32	1168.60	7.02	0.6	-0.9	-0.520	rich in H	
4	E I H P F I Q H P V H G F H R	-	X	-	AMP 2.3	NAMP 0.120	NAMP 0.041	NAMP	NAMP 0.075	232-246	1851.11	1850.96	7.19	0.9	-0.9	-0.607	rich in H	
	E V A P A P A S T G G R	-	X	-	NAMP -0.51	NAMP 0.022	NAMP 0.229	NAMP	NAMP 0.001	568-578	1041.13	1041.53	6.10	-0.1	-0.9	-0.518	putative leaky	
	G Q A A R P E V A P A P S T G G R	X	X	-	NAMP -1.05	NAMP 0.061	NAMP 0.194	NAMP	NAMP 0.062	562-578	1621.78	1621.84	9.60	0.9	0.1	-0.712	putative leaky	
	G Q A A R P E V A P A P S T G G R I V A P G H R A	-	X	-	NAMP -0.67	NAMP 0.413	NAMP 0.407	AMP	AMP 0.827	562-587	2480.78	2480.32	11.70	2.2	1.1	-0.381	putative leaky	
5	I G Q A A R P E V A P A P S T G G R	X	X	-	NAMP -0.51	NAMP 0.055	NAMP 0.300	NAMP	NAMP 0.207	561-578	1734.94	1734.92	9.60	0.9	0.1	-0.422	putative leaky	
	Q A A R P E V A P A P S T G G R	X	X	-	NAMP -1.50	NAMP 0.060	NAMP 0.111	NAMP	NAMP 0.014	563-578	1564.72	1547.79	9.60	0.9	0.1	-0.731	putative leaky	
	R P E V A P A P S T G G R	X	X	-	NAMP -0.86	NAMP 0.113	NAMP 0.113	NAMP	NAMP 0.002	566-578	1294.44	1294.69	9.60	0.9	0.1	-0.908	putative leaky	
	S T D V P R D P W V W G S A H P Q A Q H T R	-	X	-	NAMP -1.63	NAMP 0.024	NAMP 0.040	NAMP	NAMP 0.082	622-643	2528.73	2528.22	6.66	0.4	-0.9	-1.245	putative leaky	
5.3	V Q Q E V A P A R G V V G M V V P E G H R A	X	X	-	NAMP -0.61	NAMP 0.105	NAMP 0.250	NAMP	NAMP 0.077	459-481	2343.70	2343.23	6.73	0.2	-0.9	0.065	putative leaky	
	V Q Q E V A P A R G V V G M V V P E G H R A R	X	X	-	NAMP -0.88	NAMP 0.092	NAMP 0.216	NAMP	NAMP 0.099	459-482	2499.89	2499.34	9.49	1.2	0.1	-0.125	putative leaky	

**Figure 1.** Flow chart of egg white (EW) fractionation by ultrafiltration and nanofiltration for the preparation of the 10kDa, 1kDa, and 400Da egg-white filtrates (EWFs). Egg white retentates (EWRs) are the fractions retained by the membranes.

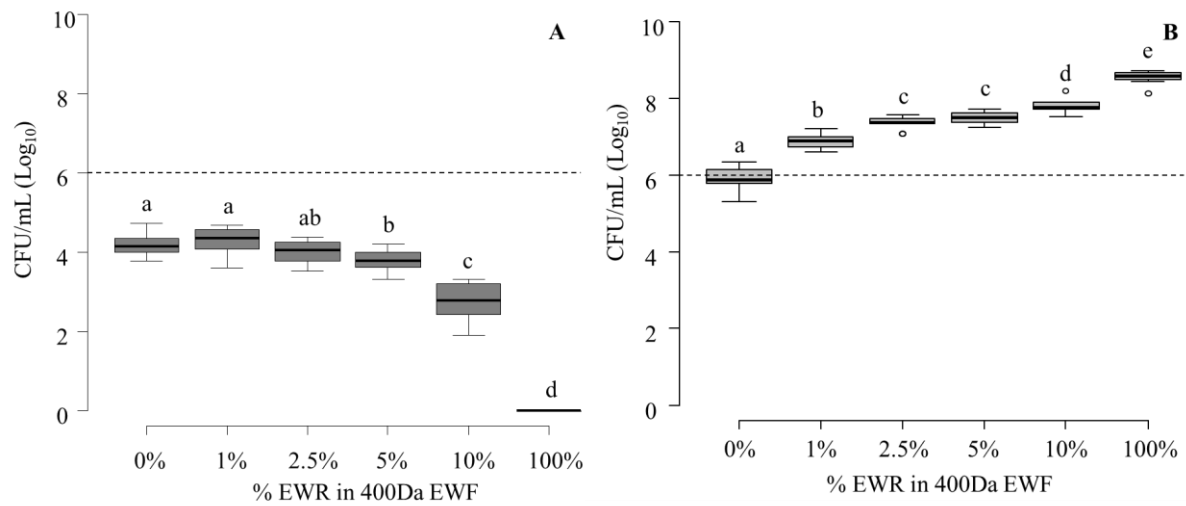


**Figure 2.** *S. Enteritidis* numeration after incubation for 24 h at 45 °C (A) and 30 °C (B) in TSB pH 7.3, egg white (EW) and 10kDa, 1kDa and 400Da egg white filtrates (EWFs). Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from nine replicates (three biological replicates, each with three technical replicates). Samples with different letters display significantly different mean values (p<0.0001 in A, p<0.001 in B).

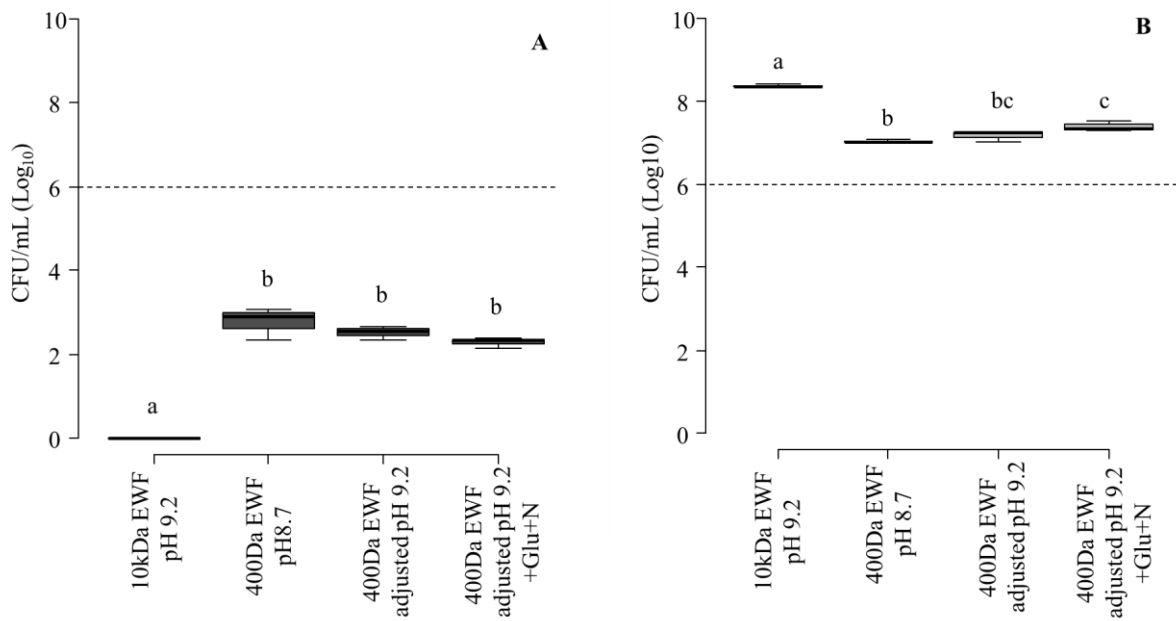




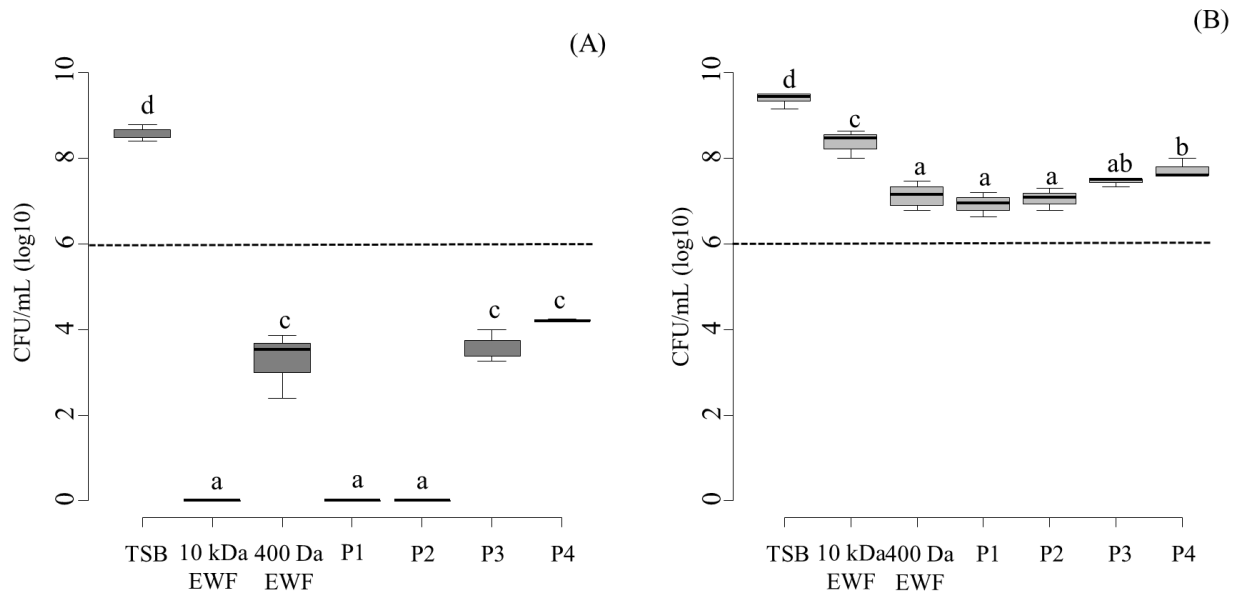
**Figure 3.** *S. Enteritidis* survival after incubation for 24 h at 45 °C (A) and 30°C (B) in 400Da egg white filtrate (EWF) supplemented with increasing levels of the 400Da egg white retentate (EWR). Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from nine replicates (three biological replicates, each with three technical replicates). Samples with different letters display significantly different mean values (p<0.001).



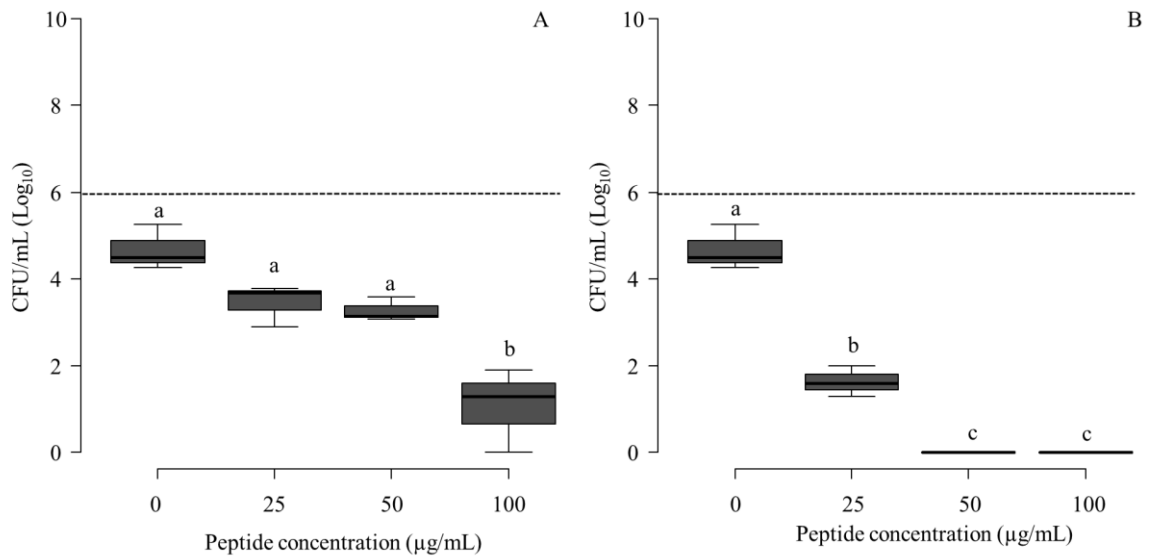
**Figure 4.** Effect of pH, glucose and nitrogen levels on *S. Enteritidis* survival in the 400Da EWF. *S. Enteritidis* was incubated for 24 h at 45 °C (A) or 30 °C (B) in 10kDa EWF at pH 9.2, in 400Da EWF at pH 8.7 and 9.2, in 400Da EWF at pH 9.2 and with addition of a nitrogen (N) source and glucose (Glu) (up to 3 mM N and 25 mM glucose). Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from three technical replicates. Samples with different letters display significantly different mean values (p<0.01).



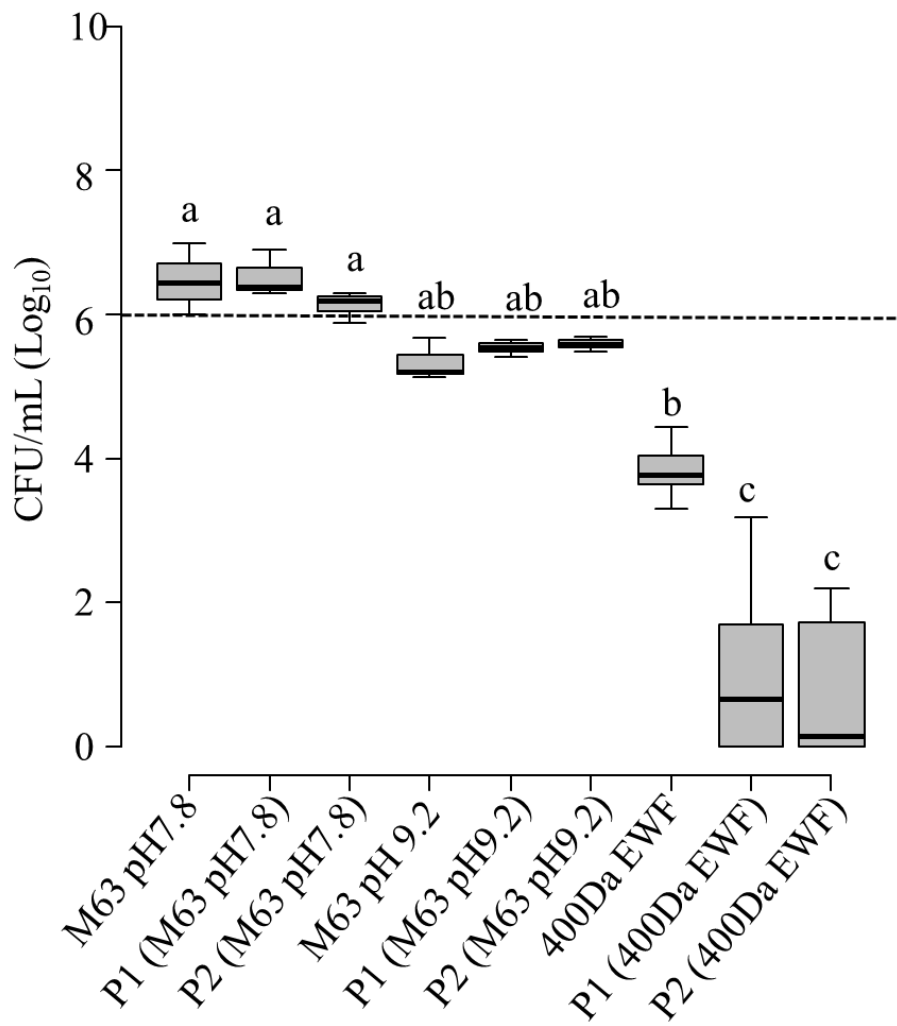
**Figure 5.** Effect of P1-P4 peptides on *S. Enteritidis* survival in 400Da EWF. *S. Enteritidis* was incubated for 24 h at 45 °C (A) and 30 °C (B) in TSB pH 7.3, 10kDa EWF, 400Da EWF, and 400Da EWF with addition of 100 µg/mL of the P1, P2, P3 or P4 synthetic peptides. Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from three technical replicates. Samples with different letters display significantly different mean values (p<0.001).



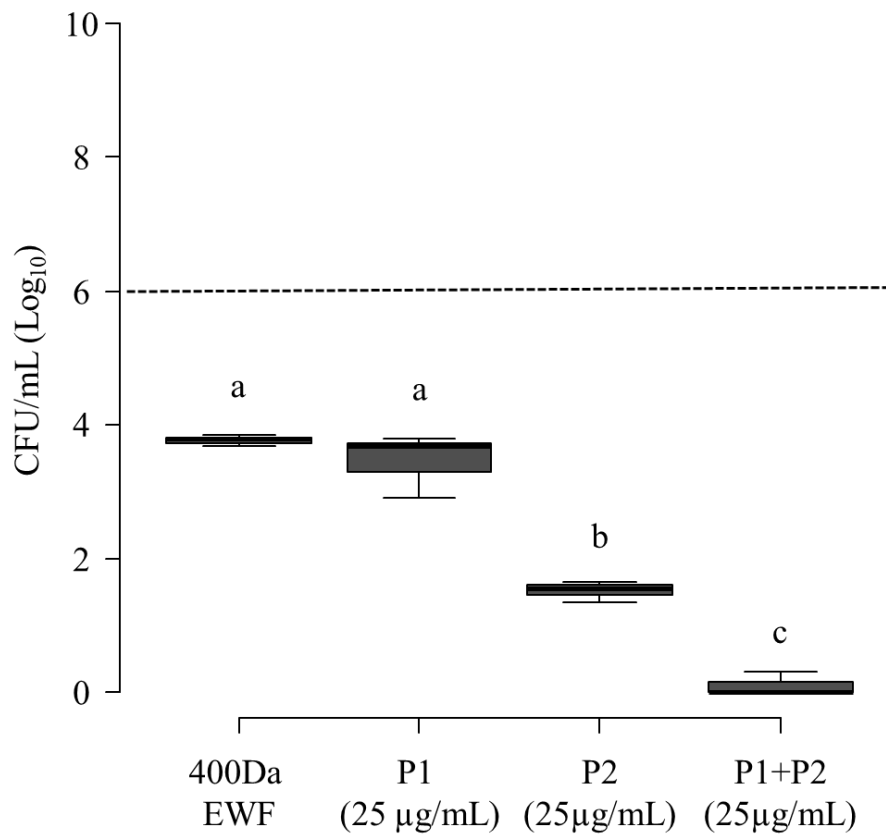
**Figure 6.** Effect of peptide concentration on the bactericidal activity of P1 and P2 against *S. Enteritidis* in 400Da EWF at 45 °C. P1 (A) and P2 (B) were added at 0 to 100 µg/mL. Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from six replicates (two biological replicates, each with three technical replicates). Samples with different letters display significantly different mean values (p<0.01 for A, and p<0.001 for B).



**Figure 7:** Effect of both AMPs P1 and P2 on *S. Enteritidis* survival in M63 minimal medium and in 400Da EWF. *S. Enteritidis* was incubated for 24 h at 45°C in M63 at pH 7.8 and 9.2, and in 400Da EWF, with or without addition of 100 µg/mL P1 or P2. Bacteria were initially inoculated at 10<sup>6</sup> CFU/mL (dotted line). Means and standard deviations were calculated from three technical replicates. Samples with different letters display significantly different mean values (p<0.001).



**Figure 8.** Synergistic bactericidal effect of the AMPs P1 and P2 against *S. Enteritidis* at 45 °C in 400Da EWF. *S. Enteritidis* was incubated for 24 h at 45 °C in 400Da EWF with addition of P1 (25 µg/mL), P2 (25 µg/mL), or P1+ P2 (12.5 µg/mL P1; 12.5 µg/mL P2). Bacteria were initially inoculated at 10<sup>6</sup>CFU/mL (dotted line). Means and standard deviations were calculated from three technical replicates. Samples with different letters display significantly different mean values (p<0.001).



# TOC Image

