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8 **Synthesis of trypsin-resistant variants of the *Listeria*-active bacteriocin**
9 **salivaricin P**

10

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29 **Abstract**

30 Two-component Salivaricin P-like bacteriocins have demonstrated potential as
31 antimicrobials capable of controlling infections in the gastrointestinal tract (GIT). The
32 anti-*Listeria* activity of salivaricin P is optimal when the individual peptides, Sln1 and
33 Sln2, are added in succession in a 1:1 ratio. However, as degradation by digestive
34 proteases may compromise the functionality of these peptides within the GIT we
35 investigated the potential to create salivaricin variants with enhanced resistance to the
36 intestinal protease, trypsin. A total of 11 variants of the salivaricin P components were
37 generated in which conservative modifications at the trypsin-specific cleavage sites
38 were explored in order to protect the peptides from trypsin degradation while
39 maintaining their potent antimicrobial activity. Analysis of these variants revealed that
40 eight were resistant to trypsin digestion while retaining antimicrobial activity.
41 Combining the complementary trypsin resistant variants Sln1-5 and Sln2-3 resulted in
42 a MIC₅₀ of 300 nM against *Listeria monocytogenes*, a 3.75-fold reduction in activity
43 compared to wild-type salivaricin P. This study demonstrates the potential of
44 engineering bacteriocins variants which are resistant to specific protease action but
45 which retain significant antimicrobial activity.

46 **Introduction**

47 Salivaricin P is a two-component class IIb bacteriocin produced by the porcine
48 intestinal isolate *Lactobacillus salivarius* DPC6005, whose target specificity includes
49 the food-borne intestinal pathogen *Listeria monocytogenes* (2). Production of
50 salivaricin P-like bacteriocins is a common feature amongst *Lactobacillus salivarius*
51 strains of intestinal origin (2) and it is evident that these bacteriocins may play an
52 important role within the gut. This is demonstrated by the *in vivo* protection provided
53 against *Listeria monocytogenes* infection by the salivaricin P-like bacteriocin, abp118
54 (5). Production of salivaricin P is also likely to be responsible for the dominance of
55 *Lb. salivarius* DPC6005 within the porcine ileum following its co-administration with
56 four other probiotics (33). Despite this, there would seem to be potential to further
57 augment salivaricin P activity in the gut as, following oral administration of the *Lb.*
58 *salivarius* DPC6005 to mice, it was possible to detect the strain, but not the
59 bacteriocin, in murine caecal samples (O’Shea, unpublished data).

60 The proteinacious nature of bacteriocins makes them vulnerable to proteolysis
61 by gut-associated proteases. This can be regarded as a beneficial trait in that it helps to
62 ensure that bacteriocins used as food preservatives don’t negatively impact the natural
63 gut microbiota. Conversely, it may compromise the efficacy of narrower spectrum
64 bacteriocins deployed to control gastrointestinal tract (GIT) infections. As a result,
65 approaches to improve the delivery and bioavailability of bacteriocins within the GIT
66 have been under investigation. One option is encapsulation. Indeed encapsulated OR-
67 7, a bacteriocin produced by *Lb. salivarius* NRRL B-30514, has demonstrated
68 bioactivity *in vivo* and successfully reduced *Campylobacter* colonization in poultry
69 (30). Theoretically, engineering of a bacteriocin to reduce its sensitivity to proteases is
70 also a viable option. Engineering of bacteriocins has been used to generate variants

71 which have helped scientists to better understand structure-function relationships of
72 these peptides (6, 7, 10, 11, 14, 24, 25, 28, 31, 34), and there have been recent
73 successes in which the antimicrobial activity of bacteriocins has been enhanced
74 through a bioengineering-based approach (1, 8, 9, 15, 17, 19, 35). Despite this,
75 bioengineered peptides with enhanced protease-resistance have only been generated in
76 the case of the class I bacteriocin, gallidermin. Unfortunately, however, the alterations
77 that enhanced the protease resistance of two gallidermin analogues were found to be
78 detrimental with respect to the associated antimicrobial activity (26) . In this study we
79 sought to generate engineered bacteriocins that are better suited to resisting the harsh
80 environment of the GIT, without compromising their antimicrobial activity. More
81 specifically, our aim was to increase the resistance of the two component peptides of
82 salivaricin P, Sln1 and Sln2 (both required for optimal antimicrobial activity), to
83 proteolysis by the gastric protease trypsin, without significantly reducing their
84 antimicrobial activity. Trypsin specifically cleaves peptides at the carboxyl side of
85 lysine and arginine residues, unless followed by a proline (18). Consequently, a series
86 of variants of both the Sln1 and Sln2 peptides were designed to potentially resist
87 trypsin digestion as a result of incorporating amino acid substitutions at trypsin
88 specific cleavage sites or insertion of proline residues following these sites. In
89 addition to creating a number of engineered peptides which provide an important
90 insight into the structure-function relationships of these peptides, we were successful
91 in generating a variety of trypsin resistant derivatives of both peptides which retain
92 significant antimicrobial potency. This represents the first example of the use of
93 peptide engineering to specifically target and create trypsin resistant bacteriocins.

94

95 **Materials and Methods**

96 **Bacterial strains and culture conditions.** *Lactobacillus salivarius* DPC6005
97 and *Lactobacillus bulgaricus* LMG 6901 were routinely grown in MRS (Difco
98 Laboratories, Detroit, MI) at 37°C anaerobically. *Listeria innocua* DPC3572, *Listeria*
99 *monocytogenes* NCTC 11994 and *Enterococcus faecalis* DPC1142 were grown in
100 M17 (Difco) supplemented with 0.5% glucose (GM17) at 37°C aerobically, and
101 GM17 supplemented with 0.1% [vol/vol] Tween 80 (Sigma, Poole, Dorset, UK) was
102 used for the microtiter plate assay system.

103

104 **Synthesis and purification of the salivaricin P peptides.** Natural salivaricin
105 P Sln1 and Sln2 peptides were purified from an overnight culture of *Lb. salivarius*
106 DPC6005 as described previously (2). The salivaricin P peptides were also
107 synthesised according to the amino acid sequence reported by Barrett et al., 2007 (2)
108 using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a
109 CEM LibertyTM microwave peptide synthesiser using a H-Leu-HMPB-ChemMatrix®
110 resin and a H-His (Trt)-HMPB-ChemMatrix® resin (PCAS Biomatrix Inc. Quebec,
111 Canada) for Sln1 and Sln2 (and corresponding variants), respectively. The
112 antimicrobial activity of the peptides was confirmed by well diffusion assay as
113 previously described (29). The synthetic peptides were purified by RP-HPLC using a
114 Jupiter C5 (10u 300Å) column (Phenomenex, Cheshire, United Kingdom) developed
115 in a gradient from 25% (vol/vol) acetonitrile containing 0.1% trifluoroacetic acid
116 (TFA) to 50% (vol/vol) acetonitrile containing 0.1% TFA from 10 to 40 min at a flow
117 rate of 3.5 ml/min. Absorbance was monitored at a wavelength of 214 nm. Fractions
118 containing peptides with the desired molecular mass, identified using Matrix-assisted
119 laser desorption ionisation-time of flight (MALDI TOF) mass spectrometry (MS),
120 were pooled and lyophilised using a Genevac HT 4X (Genevac Ltd. Ipswich, United

121 Kingdom). The peptides were dissolved in 70% (vol/vol) isopropanol at a
122 concentration of 5 mg/ml and stored at -20°C under nitrogen. Appropriate dilutions of
123 the peptides in 50 mM sodium phosphate buffer were used for bacteriocin assays.

124

125 **Specific activity determination.** A microtiter plate assay system was used to
126 determine the minimum concentration of salivaricin P required to inhibit growth of
127 the indicator, *L. innocua* DPC3572 by 50% (MIC₅₀). Each plate included triplicate
128 assays at each concentration examined. Each well contained a total volume of 200 µl,
129 comprised of purified Sln1 and purified Sln2 (or variants thereof), and 150 µl of a 1-
130 in-10 dilution of the indicator culture (A_{590} of 0.1) in GM17 broth (supplemented with
131 0.1% (vol/vol) Tween 80). Control wells contained media only (blanks), untreated
132 indicator culture or the indicator treated with Sln1 or Sln2 alone. The microtiter plate
133 cultures were then incubated for 6 h at 37°C and the optical density at 590 nm (OD₅₉₀)
134 was recorded at 0 h and 6 h (GENios plus; TECAN, Switzerland). Triplicate readings
135 were averaged and blanks were subtracted from these readings. The amount of
136 bacteriocin that inhibited the indicator strain by 50% was defined as 50% of the final
137 OD₅₉₀ ± 0.05 of the untreated control culture. The individual concentrations of Sln1
138 and Sln2, which in combination inhibited the growth of *L. innocua* DPC3572 by 50%,
139 were plotted as an isobologram. The point of intersection of the concentration of Sln1
140 and Sln2 determines the specific activity of the peptides as well as the optimal peptide
141 ratio for activity.

142

143 **Sequential treatment of *L. innocua* DPC3572 with salivaricin P**
144 **component peptides.** The sequential Sln1 and Sln2 treatment of the indicator *L.*
145 *innocua* DPC3572, and vice versa, was assayed similarly to the procedure described

146 by Morgan et al., 2005 (21). The *L. innocua* cultures (in triplicate) were diluted 1 in
147 10 and 150 µl of each culture was added to 500 µl microfuge tubes which contained
148 Sln1 or Sln2 individually (at concentrations of 0 nM, 40 nM, 80 nM, 120 nM, 160 nM
149 and 200 nM). Tubes were left at room temperature for approximately 20 min (to
150 enable binding of peptide molecules to cell surfaces) prior to centrifuging at 13,000 ×
151 g for 30 seconds. Supernatants were removed from each tube and cell pellets were
152 washed twice with GM17 broth (supplemented with 0.1% (vol/vol) Tween 80). Cell
153 pellets were resuspended in 150 µl of fresh broth. Cells that had been exposed to Sln1
154 alone were added to microtiter wells which contained Sln2, and cells that had been
155 exposed to Sln2 alone were added to microtiter wells which contained Sln1 (at
156 concentrations of 0 nM, 40 nM, 80 nM, 120 nM, 160 nM and 200 nM). Microtiter
157 plates were incubated at 37°C and read at hourly intervals for 6 hours, with the first
158 reading representing time zero. Controls included expos cells to Sln1 and Sln2 in
159 combination.

160

161 **Activity of trypsin resistant bacteriocin variants.** Sln1, Sln2 and their
162 respective variants were digested using trypsin gold (Promega Corporation, Madison,
163 USA) according to the manufacturer's instructions. Digestion of the peptides was
164 confirmed by MALDI-TOF MS analysis. The MIC₅₀ of each variant was assessed by
165 combining with the wild-type peptide using the microtiter based assay system
166 described above. The variants displaying lowest MIC₅₀ when combined with the wild-
167 type complementary peptide were then similarly combined with one another to
168 determine their specific antilisterial activity.

169

170 **Results**

171 **Sln1 and Sln2 are active at nanomolar concentrations in an optimal ratio**
172 **of 1:1.** The salivaricin P peptides, Sln1 and Sln2, were generated by peptide synthesis.
173 MS analysis confirmed that the masses of the synthetic peptides were identical to
174 those produced by *Lb. salviarius* DPC6005 and antimicrobial activity assays using
175 *Listeria* as the indicator established that the natural and synthetic peptides have equal
176 potency. The combined concentrations of Sln1 and Sln2 that were required to inhibit
177 the growth of the indicator strain, *L. innocua* DPC3572, by 50% were plotted as an
178 isobologram (Fig. 1). As is the case for another class IIb bacteriocin, lactococcin G
179 (22), the addition of Tween 80 to the culture medium (final concentration, 0.1%
180 [vol/vol]) increased the assay sensitivity almost 10-fold (data not shown), and thus
181 was incorporated in all cases. When assessed individually both Sln1 and Sln2
182 displayed activity at micromolar concentrations against *L. innocua* DPC3572.
183 However, Sln1 (MIC₅₀ of 5M) has ten-fold higher individual activity than Sln2
184 (MIC₅₀ of 50 M). Studies with combinations of these peptides revealed that activity
185 was optimal when Sln1 and Sln2 were combined at equal concentrations. This optimal
186 ratio is consistent with that reported when the synthetic components of salivaricin
187 CRL1328, with which salivaricin P shares 95% identity (Fig. 2), were tested using an
188 *Enterococcus faecalis* indicator strain (32). Here we also establish that when Sln1 and
189 Sln2 are combined the corresponding MIC₅₀ value is 50 nM (Fig. 1), one hundred-fold
190 more active than Sln1 alone.

191

192 **Salivaricin P peptides act sequentially to kill target cells at nanomolar**
193 **concentrations.** The Sln1 and Sln2 peptides were subjected to further investigation in
194 order to determine whether or not they function in a sequential manner. It was
195 apparent that growth inhibition was not observed when *L. innocua* DPC3572 was first

196 exposed to Sln1, followed by extensive washing and subsequent addition of Sln2.
197 However, when the indicator was pre-treated with Sln2, prior to extensive washing
198 and subsequent addition of Sln1, the degree of inhibition was comparable to that
199 achieved when both peptides were added simultaneously (Fig. 3). This confirms a
200 sequential mode of action and suggests that Sln2 is likely to be the receptor binding
201 component of the bacteriocin.

202

203 **Design of salivaricin P variant peptides.** Trypsin is predicted to specifically
204 cleave at two locations in Sln1, i.e. after both Lys1 and Arg2, which would result in a
205 43 amino acid (aa) product with a molecular mass of 3812 Da. It is also predicted to
206 cleave at two locations in Sln2, after Lys1 and Arg10, which would result in a 9 aa
207 fragment of 880 Da and 36 aa fragment of 3293 Da. These predictions were
208 confirmed by MALDI-TOF MS analysis of the peptides before and after tryptic
209 digestion (Table 2), and the effect of trypsin digestion on the activity of salivaricin P
210 is presented in Figure 4. The residues that are lost as a consequence of trypsin
211 digestion are, due to their cationic nature, likely important for bacteriocin activity. In
212 both class I and class IIa bacteriocins these residues have frequently been found to
213 play an important role in mediating the initial interaction with the anionic target cells
214 via electrostatic interactions (3, 4, 17). Indeed, recent studies with the most
215 extensively studied class IIb bacteriocin, lactococcin G, also revealed an important
216 role for such residues in positioning the cationic C-termini of the bacteriocin
217 components inside the target cell (23, 25, 27). Here we sought to maintain the potent
218 antilisterial activity of the salivaricin P components while establishing a trypsin
219 resistant phenotype. Therefore, to preserve the function of the positively charged
220 trypsin-specific residues of salivaricin P, a variety of conservative changes to Sln1

221 and Sln2 were investigated (Table 1). These alterations included the removal and/or
222 replacement by histidine of the trypsin-sensitive Lys and Arg residues as well as the
223 insertion of proline residues adjacent to trypsin-specific sites to hinder the protease
224 activity. Initially, the creation of Sln1-1, which lacks an N-terminal Lys, was
225 synthesized in order to reveal the impact of partial trypsin digestion on the specific
226 anti-*Listeria* activity of Sln1. A number of other peptides were also designed with a
227 view to enhancing trypsin resistance. These included Sln1-2, a variant of Sln1
228 containing a His rather than Lys1-Arg2 at the N-terminus, as well as Sln1-3 and Sln1-
229 4, both of which contain His residues in place of either Lys1 or Arg2 in addition to the
230 insertion of a proline residue adjacent to the other trypsin-sensitive residue. Sln1-5
231 was also designed to include an additional Pro residue after the N-terminal Lys,
232 together with the removal of a glycine such that both trypsin-sensitive residues are
233 bordered at the carboxyl side by a proline. With respect to Sln2, a variant which
234 contained an Arg10His substitution, Sln2-1, was predicted to confer trypsin resistance
235 at this location in Sln2. However, this variant retains a trypsin cleavage site at Lys1,
236 digestion of which is predicted to result in a 45 aa product of 4137 Da. Sln2-2 and
237 Sln2-3 were designed to address this by combining the Arg10His substitution with a
238 Pro insertion at the carboxyl side of Lys1 or a Lys1His substitution, respectively. Two
239 truncated derivatives of Sln2 were also synthesized, Sln2-4, consisting of the 10 N-
240 terminal aa fragment cleaved upon tryptic digestion of Sln2, and Sln2-5, which lacks
241 the nine N-terminal amino acids of Sln2 but contains a Pro insertion following the
242 now N-terminally located Arg residue.

243

244 **Activity and protease resistance of Sln1 variants.** The variants described
245 above were each synthesized and purified. The activity was assessed against *L.*

246 *innocua* DPC3572 by combining each variant in equimolar concentrations with the
247 wild-type version of the complementary peptide (Table 1). MS analysis was
248 performed to assess the trypsin sensitivity of each of the synthetic variants (Table 2).
249 This revealed that the purified Sln1 peptide and variants thereof are very sensitive to
250 oxidation (as demonstrated by an associated 16 Da increase in the molecular mass of
251 the peptide) which negatively impacted on antimicrobial activity. Studies with Sln1-1
252 revealed that the N-terminal Lys of the peptide is not essential as the MIC₅₀ of Sln1-1,
253 when combined with Sln2, was 80 nM. However, as expected, this peptide remains
254 susceptible to tryptic digestion, which results in a 3812 Da cleavage product (Table
255 2). When this change was coupled with an Arg2His alteration to generate the trypsin
256 resistant variant Sln1-2 (Table 2), the corresponding peptide was now four-fold less
257 active (MIC₅₀ of 200 nM) than Sln1 (MIC₅₀ of 50 nM), when combined with Sln2.
258 Crucially, however, this peptide had a trypsin resistant phenotype. These MIC₅₀ values
259 were further improved to 100 nM and 130 nM in the trypsin resistant peptides Sln1-3
260 and Sln1-4, respectively (Table 2). However, of all the trypsin-resistant Sln1 variants,
261 Sln1-5 was most impressive in that its potency against *L. innocua* DPC3572 is
262 comparable to that of wild type Sln1, exhibiting a MIC₅₀ of 80 nM (when combined
263 with Sln2).

264 Finally, as noted above, the Sln1 peptide and its variants are susceptible to
265 oxidation. This phenomenon has previously been associated with a number of other
266 class II bacteriocins (13, 16, 20) and has been attributed to the oxidation of the
267 sulphur atom of methionine residues resulting in the formation of methionine
268 sulfoxide. To determine if the oxidation of Sln1 is due to the methionine residue at
269 position 37 an additional Sln1 variant, Sln1-6, which contains a Met37Leu
270 substitution, was generated. Although the activity of this variant was reduced by more

271 than two-fold (MIC₅₀ of 120 nM), compared to that of the wild type, thereby
272 indicating the importance of this methionine residue for activity, this variant did not
273 display susceptibility to oxidation, confirming that the Met37 residue is the oxidation-
274 sensitive residue.

275

276 **Activity and protease resistance of Sln2 variants.** Trypsin also cleaves at
277 two sites within Sln2 resulting in the removal of Lys1 as well as a further nine aa N-
278 terminal fragment upon cleavage at Arg10 (Table 2). Substitution of Sln2Arg10 with
279 His (Sln2-1) resulted in a four-fold decrease in activity when combined with wild type
280 Sln1 (Table 1). An associated cleavage product of 4137 Da upon tryptic digestion
281 (Table 2) confirmed trypsin resistance at this position. When this modification was
282 coupled with the insertion of a Pro residue following Lys1 (Sln2-2), complete trypsin
283 resistance was achieved without further reducing antimicrobial activity (Tables 1 and
284 2). However, substituting Lys1 as well as Arg10 with His (Sln2-3) also resulted in
285 trypsin resistance (Table 2) and led to an improvement in specific activity (MIC₅₀ of
286 120 nM), when combined with wild-type Sln1. The 10 N-terminal aa fragment of Sln2
287 (Sln2-4) did not display anti-*Listeria* activity. However, although the specific activity
288 of the trypsin resistant Sln2-5 variant (Table 2) was reduced five-fold relative to Sln2
289 (again when combined with Sln1; Table 1), it retained a similar spectrum of activity,
290 inhibiting species of *Lactobacillus*, *Enterococcus* and *Listeria* (as determined by well
291 diffusion assay; Fig. 5). It is thus apparent that the nine N-terminal residues of Sln2
292 are not essential for peptide function.

293

294 **Specific activity of combined trypsin resistant variants (Sln1-5 and Sln2-**
295 **3).** The specific activity determinations using a combination of the two most potent,

296 trypsin-resistant peptides, Sln1-5 and Sln2-3, revealed that their combined specific
297 activity (MIC₅₀ of 300 nM) was just 3.75-fold less than that of the wild type peptides
298 (80 nM) against *L. monocytogenes* NCTC 11994 (Fig. 6).

299

300 **Discussion**

301 The naturally occurring two component bacteriocins, salivaricin P, abp118 and
302 salivaricin CRL1328 share greater than 95% homology (2, 12, 32). While these
303 bacteriocins have shown promise in controlling intestinal pathogens *in situ*, a
304 fundamental understanding of how these peptides function is crucial, both in terms of
305 gaining regulatory approval for such applications and to assist in the design of
306 antimicrobial derivatives with improved functionality. Recent studies revealed the
307 pore forming ability of salivaricin CRL1328, and thus presumably of salivaricin P and
308 abp118, results in dissipation of both the transmembrane electrical potential and the
309 pH gradient of sensitive cells (32). While the synthetic salivaricin P components
310 display weak individual activity, this study confirmed that optimal synergistic
311 antimicrobial activity is achieved when the component peptides are combined at
312 nanomolar concentrations in a ratio of 1:1. We also demonstrated that the individual
313 peptides function in a sequential manner and it is established that Sln2, but not Sln1,
314 is capable of binding to the target cell membrane in the absence of its companion
315 peptide. While it is likely that this involves an interaction with a docking molecule or
316 receptor, studies of Sln2 peptides with altered chirality will be required to verify the
317 existence of a cellular receptor for Sln2. The subsequent addition of Sln1, to
318 potentially form a bacteriocin-membrane receptor complex, is then required for
319 optimal activity.

320 A variety of alterations of each of the component peptides were designed,
321 created and assessed with a view to enhancing the trypsin-resistance of salivaricin P
322 without dramatically reducing the antimicrobial activity of the bacteriocin. It was
323 noted that, in general, insertion of proline residues represented a very well tolerated
324 means of generating trypsin-resistant salivaricin variants. The least deleterious Sln1
325 variant was that in which both trypsin-sensitive residues were present (Sln1-5), with
326 adjacent proline residues introduced to confer trypsin resistance. While a comparable
327 Sln2 variant was not generated, it was noted that an additional proline insertion in
328 Sln2-2 did not further reduce its activity relative to Sln2-1, which like Sln2-2 also
329 contained an Arg10His substitution. In contrast, the most deleterious mutation of Sln1
330 involved a single histidine substitution of both trypsin-sensitive residues (Sln1-2).
331 Conversely, the conservative histidine substitution of both trypsin-sensitive residues
332 of Sln2 was well tolerated, with the corresponding variant, Sln2-3, being the most
333 active of all of the Sln2 variants. Assays with purified Sln1 peptides revealed a
334 consistent tendency for the peptides to become oxidised. It was established that this
335 oxidation occurred at Met37 and was absent in a Met37Leu variant of Sln1. The
336 reduced activity of the corresponding Sln1-6 peptide revealed that the methionine is
337 required for optimal activity, perhaps being required to facilitate correct peptide
338 folding or for the interaction of Sln1 with Sln2 or the target cell membrane.

339 Analysis of the trypsin-resistant N-terminally truncated derivative of Sln2,
340 Sln2-5, revealed that while these changes were deleterious, the truncated variant
341 continued to exhibit anti-listerial activity at nanomolar concentrations and possessed a
342 similar spectrum of inhibition to Sln2, when combined with Sln1. This finding is
343 significant given that, on the basis of the sequential action studies, it is necessary for
344 Sln2 to first bind to target cells and it is thus likely to be responsible for determining

345 target cell specificity. Interestingly, the natural salivaricin P variant, abp118, differs
346 from Sln2 with respect to two C-terminal amino acid changes (Ala43Thr and
347 His46Arg). The fact that the spectrum of inhibition of salivaricin P and abp118 also
348 differ (2), suggests the potential involvement of the C-terminal region of this peptide
349 in determining target cell specificity. Further alterations of the C-terminal residues of
350 Sln2 will be investigated to address this. Significantly, however, the variants
351 generated here demonstrate that a variety of mutations can result in the creation of
352 trypsin-resistant variants of the salivaricin P peptides without significantly altering the
353 length, net charge and, most importantly, potency of the bacteriocin.

354 Bacteriocins have great potential as antimicrobials for biopreservation and
355 biomedicine-related applications. The inactivation of bacteriocins used as food
356 biopreservatives by digestive enzymes, thereby preventing a detrimental impact on the
357 commensal gut microbiota, is considered desirable. However, there are also instances
358 where targeted antimicrobial activity in the gut is desirable, for example to inhibit
359 specific pathogenic microorganisms. While strategies such as bioencapsulation and
360 the exploitation of probiotics are attractive options to facilitate the *in situ* production
361 of bacteriocins (as with the abp118 and salivaricin P producers *Lb. salivarius*
362 UCC118 and *Lb. salivarius* DPC6005, respectively (5, 33)), the inherent protease
363 sensitivity of these peptides remains an issue. In this study we demonstrate the
364 potential to enhance the protective function of such salivaricin P-like bacteriocins.
365 While we acknowledge that the trypsin-resistant variants described in this study will
366 remain sensitive to other proteases encountered in the GIT, they represent an
367 important step forward in that it is now apparent that such beneficial traits can be
368 incorporated in a deliberate manner. These developments can in turn be incorporated
369 into the producing strain by site-directed mutagenesis for assessment of their efficacy

370 *in vivo*, resulting in the ultimate creation of protease-resistant anti-*Listeria*
371 antimicrobials with an associated bacterial-based delivery system.

372

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378

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507 **Figure Legends**

508 **Figure 1.** Concentrations of Sln1 and Sln2 required to inhibit the growth of the
509 indicator strain, *Listeria innocua* DPC3572, by 50%.

510

511 **Figure 2.** Alignment of the two component peptides of the natural bacteriocin variants
512 salivaricin P (Sln1 and Sln2) (2), abp118 (Abp118 α and Abp118 β) (12) and
513 salivaricin CRL1328 (Sal α and Sal β) (32). Conservation of amino acid residues is
514 indicated by * beneath the residue. Residues which are not conserved are underlined.
515 The component peptides of abp118 and salivaricin CRL1328 are 100% identical. Sln1
516 shares 100% identity with the corresponding peptides of abp118 and salivaricin
517 CRL1328 while Sln2 shares 95% identity with its abp118 and salivaricin CRL1328
518 counterparts.

519

520 **Figure 3.** Effects of sequential addition of the Sln1 and Sln2 peptides on the growth
521 of the indicator strain, *Listeria innocua* DPC3572 (A) Sln1 followed by Sln2 addition;
522 (B) Sln2 followed by Sln1 addition, and (C) Sln1 and Sln2 simultaneously.
523 Concentrations of 0 nM (Δ), 40 nM (x), 80 nM (\bullet), 120 nM (\blacktriangle), 160 nM (\diamond), and 200
524 nM (\square) were used. Error bars represent standard deviations on triplicate data.

525

526 **Figure 4.** The antimicrobial activity of salivaricin P (A) before and (B) after a 3 hour
527 trypsin digestion reaction, against *Listeria innocua* DPC3572. (A) The top left well
528 contains Sln1 alone, the top right well contains Sln2 alone and the bottom well
529 contains Sln1 and Sln2 combined in equal amounts. (B) The top left well contains
530 Sln1 treated with trypsin, the top right well contains Sln2 treated with trypsin and the
531 bottom well contains trypsin treated Sln1 and Sln2 combined in equal amounts.

532

533 **Figure 5.** Individual and combined antimicrobial activity of Sln1 and Sln2-5. In each
534 panel the top left well contains Sln1 alone, the top right well contains Sln2-5 alone
535 (both at 50 μ M concentrations) and the bottom well contains Sln1 and Sln2-5
536 combined in equal amounts against (A) *E. faecalis* DPC1142, (B) *Lb. bulgaricus*
537 LMG 6901 and (C) *L. innocua* DPC3572.

538

539 **Figure 6.** Effect of (A) wild-type salivaricin P at concentrations of 0 nM to 250 nM
540 and (B) trypsin resistant Sln1-5 and Sln2-3 variants at concentrations of 0 nM to 500
541 nM against *Listeria monocytogenes* NCTC11994.

542 **Table 1.** Sequences and MIC₅₀ of the salivaricin P component peptides and their
 543 variants against *L. innocua* DPC3572

| Peptide | Amino acid sequence ^a | Length (aa) | MIC ₅₀ ^b (nM) |
|---------|--|-------------|-------------------------------------|
| Sln1 | K R GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 45 | 50 |
| Sln1-1 | R GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 44 | 80 |
| Sln1-2 | H GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 44 | 200 |
| Sln1-3 | KPH GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 46 | 100 |
| Sln1-4 | HRP GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 46 | 130 |
| Sln1-5 | KPR PNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGMVGGALTCL | 45 | 80 |
| Sln1-6 | K R GPNCVGNFLGGLFAGAAAAGVPLGPAGIVGGANLGLVGGALTCL | 45 | 120 |
| Sln2 | K NGYGGSGNR WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH | 46 | 50 |
| Sln2-1 | K NGYGGSGNH WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH | 46 | 200 |
| Sln2-2 | KPNGYGGSGNH WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH | 47 | 200 |
| Sln2-3 | H NGYGGSGNH WVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH | 46 | 120 |
| Sln2-4 | K NGYGGSGNR | 10 | - |
| Sln2-5 | RPWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH | 38 | 250 |

^aThe carboxyl side of lysine (K) and arginine (R) represent trypsin specific cleavage sites.

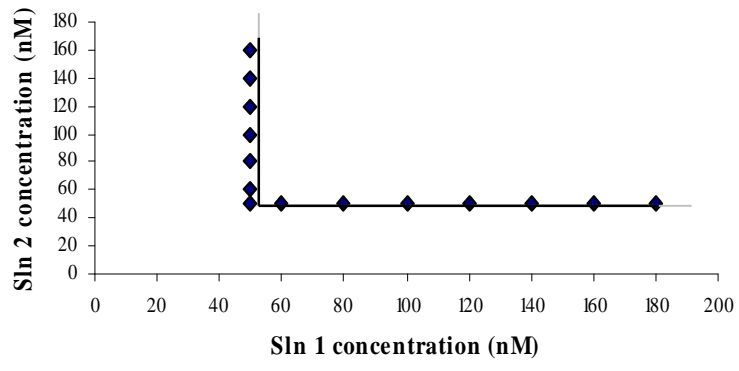
^bMIC₅₀ of bacterioicin variant combined with wild type complementary peptide.

544 **Table 2.** Impact of tryptic digestion of Sln1 and Sln2 and their respective variants as
 545 determined by mass spectrometry

| Peptide | Molecular Mass (Da) | Mass (Da) following 3 hr trypsin digest | Trypsin sensitivity^a |
|----------------|----------------------------|--|--|
| Sln1 | 4,096 | 3,812 | S |
| Sln1-1 | 3,968 | 3,812 | S |
| Sln1-2 | 3,949 | 3,949 | R |
| Sln1-3 | 4,174 | 4,174 | R |
| Sln1-4 | 4,202 | 4,202 | R |
| Sln1-5 | 4,136 | 4,136 | R |
| Sln1-6 | 4,078 | 3,812 | S |
| Sln2 | 4,284 | 880, 3,293 | S |
| Sln2-1 | 4,265 | 4,137 | S |
| Sln2-2 | 4,362 | 4,362 | R |
| Sln2-3 | 4,274 | 4,274 | R |
| Sln2-4 | 1,009 | 880 | S |
| Sln2-5 | 3,547 | 3,547 | R |

546 ^a S, sensitive; R, resistant.

547



548

549 **Figure 1.**

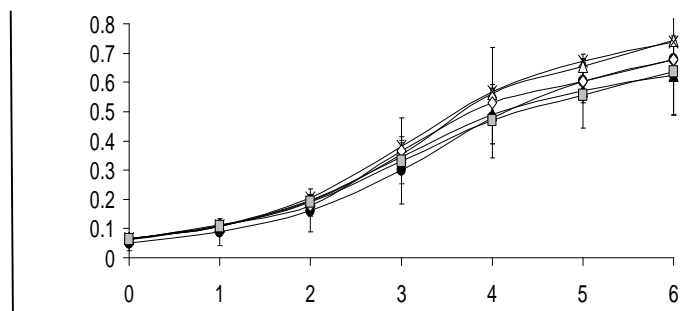
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550 Sln1 KRGPNVCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL
551 Abp118α KRGPNVCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL
552 Salα KRGPNVCVGNFLGGLFAGAAAGVPLGPAGIVGGANLGMVGGALTCL
553 *****
554
555 Sln2 KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASCH
556 Abp118β KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
557 Sal β KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
558 ***** **

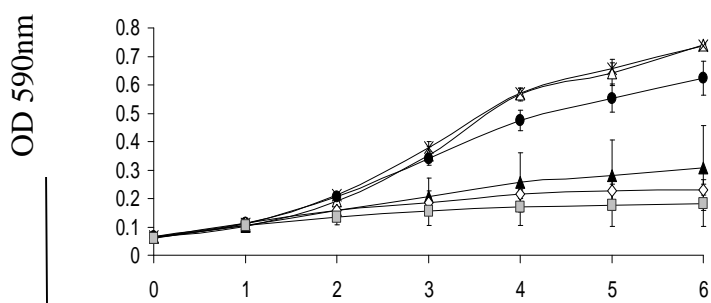
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559 **Figure 2.**

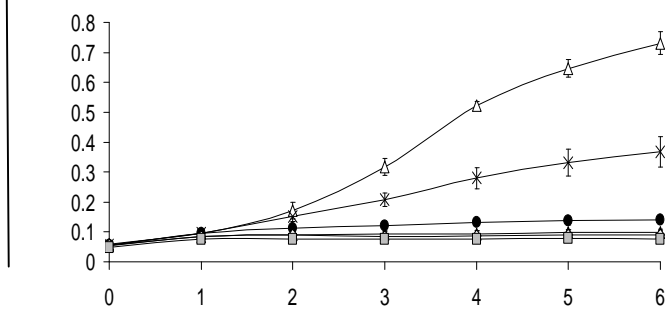
560 (A)



561
562 (B)



563
564 (C)



565
566
567

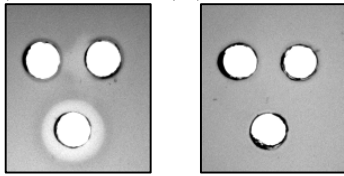
Time (hours)

568 **Figure 3.**

569

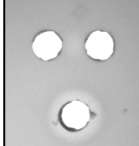
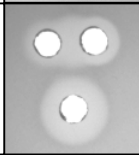
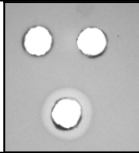
(A)

(B)



570
571

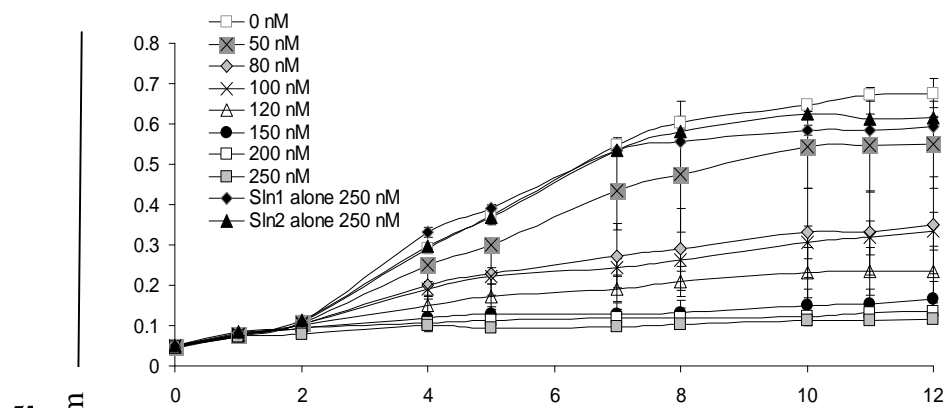
572 **Figure 4.**

| Indicator strain | |
|--|---|
| (A) <i>Enterococcus faecalis</i> DPC1142 |  |
| (B) <i>Lactobacillus bulgaricus</i> LMG 6901 |  |
| (C) <i>Listeria innocua</i> DPC3572 |  |

573
574

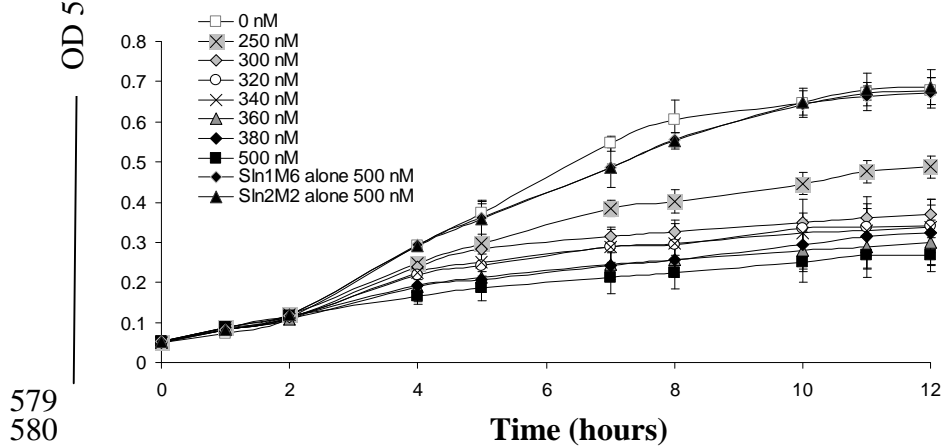
575 **Figure 5.**

576 (A)



577
578
OD 590nm

(B)



579
580
Time (hours)

581 **Figure 6.**