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

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Abstract

Two-component Salivaricin P-like bacteriocins have demonstrated potential as antimicrobials capable of controlling infections in the gastrointestinal tract (GIT). The anti-Listeria activity of salivaricin P is optimal when the individual peptides, Sln1 and Sln2, are added in succession in a 1:1 ratio. However, as degradation by digestive proteases may compromise the functionality of these peptides within the GIT we investigated the potential to create salivaricin variants with enhanced resistance to the intestinal protease, trypsin. A total of 11 variants of the salivaricin P components were generated in which conservative modifications at the trypsin-specific cleavage sites were explored in order to protect the peptides from trypsin degradation while maintaining their potent antimicrobial activity. Analysis of these variants revealed that eight were resistant to trypsin digestion while retaining antimicrobial activity.

Combining the complementary trypsin resistant variants Sln1-5 and Sln2-3 resulted in a MIC_{50} of 300 nM against Listeria monocytogenes, a 3.75-fold reduction in activity compared to wild-type salivaricin P. This study demonstrates the potential of engineering bacteriocins variants which are resistant to specific protease action but which retain significant antimicrobial activity.
**Introduction**

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

The proteinacious nature of bacteriocins makes them vulnerable to proteolysis by gut-associated proteases. This can be regarded as a beneficial trait in that it helps to ensure that bacteriocins used as food preservatives don’t negatively impact the natural gut microbiota. Conversely, it may compromise the efficacy of narrower spectrum bacteriocins deployed to control gastrointestinal tract (GIT) infections. As a result, approaches to improve the delivery and bioavailability of bacteriocins within the GIT have been under investigation. One option is encapsulation. Indeed encapsulated OR-7, a bacteriocin produced by *Lb. salivarius* NRRL B-30514, has demonstrated bioactivity in vivo and successfully reduced *Campylobacter* colonization in poultry (30). Theoretically, engineering of a bacteriocin to reduce its sensitivity to proteases is also a viable option. Engineering of bacteriocins has been used to generate variants.
which have helped scientists to better understand structure-function relationships of these peptides (6, 7, 10, 11, 14, 24, 25, 28, 31, 34), and there have been recent successes in which the antimicrobial activity of bacteriocins has been enhanced through a bioengineering-based approach (1, 8, 9, 15, 17, 19, 35). Despite this, bioengineered peptides with enhanced protease-resistance have only been generated in the case of the class I bacteriocin, gallidermin. Unfortunately, however, the alterations that enhanced the protease resistance of two gallidermin analogues were found to be detrimental with respect to the associated antimicrobial activity (26). In this study we sought to generate engineered bacteriocins that are better suited to resisting the harsh environment of the GIT, without compromising their antimicrobial activity. More specifically, our aim was to increase the resistance of the two component peptides of salivaricin P, Sln1 and Sln2 (both required for optimal antimicrobial activity), to proteolysis by the gastric protease trypsin, without significantly reducing their antimicrobial activity. Trypsin specifically cleaves peptides at the carboxyl side of lysine and arginine residues, unless followed by a proline (18). Consequently, a series of variants of both the Sln1 and Sln2 peptides were designed to potentially resist trypsin digestion as a result of incorporating amino acid substitutions at trypsin specific cleavage sites or insertion of proline residues following these sites. In addition to creating a number of engineered peptides which provide an important insight into the structure-function relationships of these peptides, we were successful in generating a variety of trypsin resistant derivatives of both peptides which retain significant antimicrobial potency. This represents the first example of the use of peptide engineering to specifically target and create trypsin resistant bacteriocins.

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

**Synthesis and purification of the salivaricin P peptides.** Natural salivaricin P Sln1 and Sln2 peptides were purified from an overnight culture of *Lb. salivarius* DPC6005 as described previously (2). The salivaricin P peptides were also synthesised according to the amino acid sequence reported by Barrett et al., 2007 (2) using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a CEM Liberty™ microwave peptide synthesiser using a H-Leu-HMPB-ChemMatrix® resin and a H-His (Trt)-HMPB-ChemMatrix® resin (PCAS Biomatrix Inc. Quebec, Canada) for Sln1 and Sln2 (and corresponding variants), respectively. The antimicrobial activity of the peptides was confirmed by well diffusion assay as previously described (29). The synthetic peptides were purified by RP-HPLC using a Jupiter C5 (10u 300Å) column (Phenomenex, Cheshire, United Kingdom) developed in a gradient from 25% (vol/vol) acetonitrile containing 0.1% trifluoroacetic acid (TFA) to 50% (vol/vol) acetonitrile containing 0.1% TFA from 10 to 40 min at a flow rate of 3.5 ml/min. Absorbance was monitored at a wavelength of 214 nm. Fractions containing peptides with the desired molecular mass, identified using Matrix-assisted laser desorption ionisation-time of flight (MALDI TOF) mass spectrometry (MS), were pooled and lyophilised using a Genevac HT 4X (Genevac Ltd. Ipswicth, United Kingdom).
Kingdom). The peptides were dissolved in 70% (vol/vol) isopropanol at a concentration of 5 mg/ml and stored at -20°C under nitrogen. Appropriate dilutions of the peptides in 50 mM sodium phosphate buffer were used for bacteriocin assays.

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

**Sequential treatment of *L. innocua* DPC3572 with salivaricin P component peptides.** The sequential Sln1 and Sln2 treatment of the indicator *L. innocua* DPC3572, and vice versa, was assayed similarly to the procedure described
by Morgan et al., 2005 (21). The *L. innocua* cultures (in triplicate) were diluted 1 in
10 and 150 μl of each culture was added to 500 μl microfuge tubes which contained
Sln1 or Sln2 individually (at concentrations of 0 nM, 40 nM, 80 nM, 120 nM, 160 nM
and 200 nM). Tubes were left at room temperature for approximately 20 min (to
enable binding of peptide molecules to cell surfaces) prior to centrifuging at 13,000 ×
g for 30 seconds. Supernatants were removed from each tube and cell pellets were
washed twice with GM17 broth (supplemented with 0.1% (vol/vol) Tween 80). Cell
pellets were resuspended in 150 μl of fresh broth. Cells that had been exposed to Sln1
alone were added to microtiter wells which contained Sln2, and cells that had been
exposed to Sln2 alone were added to microtiter wells which contained Sln1 (at
concentrations of 0 nM, 40 nM, 80 nM, 120 nM, 160 nM and 200 nM). Microtiter
plates were incubated at 37°C and read at hourly intervals for 6 hours, with the first
reading representing time zero. Controls included expos cells to Sln1 and Sln2 in
combination.

**Activity of trypsin resistant bacteriocin variants.** Sln1, Sln2 and their
respective variants were digested using trypsin gold (Promega Corporation, Madison,
USA) according to the manufacturer’s instructions. Digestion of the peptides was
confirmed by MALDI-TOF MS analysis. The MIC_{50} of each variant was assessed by
combining with the wild-type peptide using the microtiter based assay system
described above. The variants displaying lowest MIC_{50} when combined with the wild-
type complementary peptide were then similarly combined with one another to
determine their specific antilisterial activity.

**Results**
Salivaricin P peptides act sequentially to kill target cells at nanomolar concentrations. The Sln1 and Sln2 peptides were subjected to further investigation in order to determine whether or not they function in a sequential manner. It was apparent that growth inhibition was not observed when *L. innocua* DPC3572 was first
exposed to Sln1, followed by extensive washing and subsequent addition of Sln2.

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

**Design of salivaricin P variant peptides.** Trypsin is predicted to specifically cleave at two locations in Sln1, i.e. after both Lys1 and Arg2, which would result in a 43 amino acid (aa) product with a molecular mass of 3812 Da. It is also predicted to cleave at two locations in Sln2, after Lys1 and Arg10, which would result in a 9 aa fragment of 880 Da and 36 aa fragment of 3293 Da. These predictions were confirmed by MALDI-TOF MS analysis of the peptides before and after tryptic digestion (Table 2), and the effect of trypsin digestion on the activity of salivaricin P is presented in Figure 4. The residues that are lost as a consequence of trypsin digestion are, due to their cationic nature, likely important for bacteriocin activity. In both class I and class IIa bacteriocins these residues have frequently been found to play an important role in mediating the initial interaction with the anionic target cells via electrostatic interactions (3, 4, 17). Indeed, recent studies with the most extensively studied class IIb bacteriocin, lactococcin G, also revealed an important role for such residues in positioning the cationic C-termini of the bacteriocin components inside the target cell (23, 25, 27). Here we sought to maintain the potent antilisterial activity of the salivaricin P components while establishing a trypsin resistant phenotype. Therefore, to preserve the function of the positively charged trypsin-specific residues of salivaricin P, a variety of conservative changes to Sln1
and Sln2 were investigated (Table 1). These alterations included the removal and/or replacement by histidine of the trypsin-sensitive Lys and Arg residues as well as the insertion of proline residues adjacent to trypsin-specific sites to hinder the protease activity. Initially, the creation of Sln1-1, which lacks an N-terminal Lys, was synthesized in order to reveal the impact of partial trypsin digestion on the specific anti-Listeria activity of Sln1. A number of other peptides were also designed with a view to enhancing trypsin resistance. These included Sln1-2, a variant of Sln1 containing a His rather than Lys1-Arg2 at the N-terminus, as well as Sln1-3 and Sln1-4, both of which contain His residues in place of either Lys1 or Arg2 in addition to the insertion of a proline residue adjacent to the other trypsin-sensitive residue. Sln1-5 was also designed to include an additional Pro residue after the N-terminal Lys, together with the removal of a glycine such that both trypsin-sensitive residues are bordered at the carboxyl side by a proline. With respect to Sln2, a variant which contained an Arg10His substitution, Sln2-1, was predicted to confer trypsin resistance at this location in Sln2. However, this variant retains a trypsin cleavage site at Lys1, digestion of which is predicted to result in a 45 aa product of 4137 Da. Sln2-2 and Sln2-3 were designed to address this by combining the Arg10His substitution with a Pro insertion at the carboxyl side of Lys1 or a Lys1His substitution, respectively. Two truncated derivatives of Sln2 were also synthesized, Sln2-4, consisting of the 10 N-terminal aa fragment cleaved upon tryptic digestion of Sln2, and Sln2-5, which lacks the nine N-terminal amino acids of Sln2 but contains a Pro insertion following the now N-terminally located Arg residue.

Activity and protease resistance of Sln1 variants. The variants described above were each synthesized and purified. The activity was assessed against L.
innocua DPC3572 by combining each variant in equimolar concentrations with the
wild-type version of the complementary peptide (Table 1). MS analysis was
performed to assess the trypsin sensitivity of each of the synthetic variants (Table 2).
This revealed that the purified Sln1 peptide and variants thereof are very sensitive to
oxidation (as demonstrated by an associated 16 Da increase in the molecular mass of
the peptide) which negatively impacted on antimicrobial activity. Studies with Sln1-1
revealed that the N-terminal Lys of the peptide is not essential as the MIC$_{50}$ of Sln1-1,
when combined with Sln2, was 80 nM. However, as expected, this peptide remains
susceptible to tryptic digestion, which results in a 3812 Da cleavage product (Table
2). When this change was coupled with an Arg2His alteration to generate the trypsin
resistant variant Sln1-2 (Table 2), the corresponding peptide was now four-fold less
active (MIC$_{50}$ of 200 nM) than Sln1 (MIC$_{50}$ of 50 nM), when combined with Sln2.
Crucially, however, this peptide had a trypsin resistant phenotype. These MIC$_{50}$ values
were further improved to 100 nM and 130 nM in the trypsin resistant peptides Sln1-3
and Sln1-4, respectively (Table 2). However, of all the trypsin-resistant Sln1 variants,
Sln1-5 was most impressive in that its potency against $L$. innocua DPC3572 is
comparable to that of wild type Sln1, exhibiting a MIC$_{50}$ of 80 nM (when combined
with Sln2).

Finally, as noted above, the Sln1 peptide and its variants are susceptible to
oxidation. This phenomenon has previously been associated with a number of other
class II bacteriocins (13, 16, 20) and has been attributed to the oxidation of the
sulphur atom of methionine residues resulting in the formation of methionine
sulfoxide. To determine if the oxidation of Sln1 is due to the methionine residue at
position 37 an additional Sln1 variant, Sln1-6, which contains a Met37Leu
substitution, was generated. Although the activity of this variant was reduced by more
than two-fold (MIC\textsubscript{50} of 120 nM), compared to that of the wild type, thereby indicating the importance of this methionine residue for activity, this variant did not display susceptibility to oxidation, confirming that the Met37 residue is the oxidation-sensitive residue.

**Activity and protease resistance of Sln2 variants.** Trypsin also cleaves at two sites within Sln2 resulting in the removal of Lys1 as well as a further nine aa N-terminal fragment upon cleavage at Arg10 (Table 2). Substitution of Sln2Arg10 with His (Sln2-1) resulted in a four-fold decrease in activity when combined with wild type Sln1 (Table 1). An associated cleavage product of 4137 Da upon tryptic digestion (Table 2) confirmed trypsin resistance at this position. When this modification was coupled with the insertion of a Pro residue following Lys1 (Sln2-2), complete trypsin resistance was achieved without further reducing antimicrobial activity (Tables 1 and 2). However, substituting Lys1 as well as Arg10 with His (Sln2-3) also resulted in trypsin resistance (Table 2) and led to an improvement in specific activity (MIC\textsubscript{50} of 120 nM), when combined with wild-type Sln1. The 10 N-terminal aa fragment of Sln2 (Sln2-4) did not display anti-Listeria activity. However, although the specific activity of the trypsin resistant Sln2-5 variant (Table 2) was reduced five-fold relative to Sln2 (again when combined with Sln1; Table 1), it retained a similar spectrum of activity, inhibiting species of *Lactobacillus*, *Enterococcus* and *Listeria* (as determined by well diffusion assay; Fig. 5). It is thus apparent that the nine N-terminal residues of Sln2 are not essential for peptide function.

**Specific activity of combined trypsin resistant variants (Sln1-5 and Sln2-3).** The specific activity determinations using a combination of the two most potent,
trypsin-resistant peptides, Sln1-5 and Sln2-3, revealed that their combined specific
activity (MIC_{50} of 300 nM) was just 3.75-fold less than that of the wild type peptides
(80 nM) against *L. monocytogenes* NCTC 11994 (Fig. 6).

**Discussion**

The naturally occurring two component bacteriocins, salivaricin P, abp118 and
salivaricin CRL1328 share greater than 95% homology (2, 12, 32). While these
bacteriocins have shown promise in controlling intestinal pathogens in situ, a
fundamental understanding of how these peptides function is crucial, both in terms of
gaining regulatory approval for such applications and to assist in the design of
antimicrobial derivatives with improved functionality. Recent studies revealed the
pore forming ability of salivaricin CRL1328, and thus presumably of salivaricin P and
abp118, results in dissipation of both the transmembrane electrical potential and the
pH gradient of sensitive cells (32). While the synthetic salivaricin P components
display weak individual activity, this study confirmed that optimal synergistic
antimicrobial activity is achieved when the component peptides are combined at
nanomolar concentrations in a ratio of 1:1. We also demonstrated that the individual
peptides function in a sequential manner and it is established that Sln2, but not Sln1,
is capable of binding to the target cell membrane in the absence of its companion
peptide. While it is likely that this involves an interaction with a docking molecule or
receptor, studies of Sln2 peptides with altered chirality will be required to verify the
existence of a cellular receptor for Sln2. The subsequent addition of Sln1, to
potentially form a bacteriocin-membrane receptor complex, is then required for
optimal activity.
A variety of alterations of each of the component peptides were designed, created and assessed with a view to enhancing the trypsin-resistance of salivaricin P without dramatically reducing the antimicrobial activity of the bacteriocin. It was noted that, in general, insertion of proline residues represented a very well tolerated means of generating trypsin-resistant salivaricin variants. The least deleterious Sln1 variant was that in which both trypsin-sensitive residues were present (Sln1-5), with adjacent proline residues introduced to confer trypsin resistance. While a comparable Sln2 variant was not generated, it was noted that an additional proline insertion in Sln2-2 did not further reduce its activity relative to Sln2-1, which like Sln2-2 also contained an Arg10His substitution. In contrast, the most deleterious mutation of Sln1 involved a single histidine substitution of both trypsin-sensitive residues (Sln1-2).

Conversely, the conservative histidine substitution of both trypsin-sensitive residues of Sln2 was well tolerated, with the corresponding variant, Sln2-3, being the most active of all of the Sln2 variants. Assays with purified Sln1 peptides revealed a consistent tendency for the peptides to become oxidised. It was established that this oxidation occurred at Met37 and was absent in a Met37Leu variant of Sln1. The reduced activity of the corresponding Sln1-6 peptide revealed that the methionine is required for optimal activity, perhaps being required to facilitate correct peptide folding or for the interaction of Sln1 with Sln2 or the target cell membrane.

Analysis of the trypsin-resistant N-terminally truncated derivative of Sln2, Sln2-5, revealed that while these changes were deleterious, the truncated variant continued to exhibit anti-listerial activity at nanomolar concentrations and possessed a similar spectrum of inhibition to Sln2, when combined with Sln1. This finding is significant given that, on the basis of the sequential action studies, it is necessary for Sln2 to first bind to target cells and it is thus likely to be responsible for determining
target cell specificity. Interestingly, the natural salivaricin P variant, abp118, differs from Sln2 with respect to two C-terminal amino acid changes (Ala43Thr and His46Arg). The fact that the spectrum of inhibition of salivaricin P and abp118 also differ (2), suggests the potential involvement of the C-terminal region of this peptide in determining target cell specificity. Further alterations of the C-terminal residues of Sln2 will be investigated to address this. Significantly, however, the variants generated here demonstrate that a variety of mutations can result in the creation of trypsin-resistant variants of the salivaricin P peptides without significantly altering the length, net charge and, most importantly, potency of the bacteriocin.

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

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References


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**Figure Legends**

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

**Figure 2.** Alignment of the two component peptides of the natural bacteriocin variants salivaricin P (Sln1 and Sln2) (2), abp118 (Abp118α and Abp118β) (12) and salivaricin CRL1328 (Salα and Salβ) (32). Conservation of amino acid residues is indicated by * beneath the residue. Residues which are not conserved are underlined. The component peptides of abp118 and salivaricin CRL1328 are 100% identical. Sln1 shares 100% identity with the corresponding peptides of abp118 and salivaricin CRL1328 while Sln2 shares 95% identity with its abp118 and salivaricin CRL1328 counterparts.

**Figure 3.** Effects of sequential addition of the Sln1 and Sln2 peptides on the growth of the indicator strain, *Listeria innocua* DPC3572 (A) Sln1 followed by Sln2 addition; (B) Sln2 followed by Sln1 addition, and (C) Sln1 and Sln2 simultaneously. Concentrations of 0 nM (Δ), 40 nM (x), 80 nM (●), 120 nM (▲), 160 nM (◊), and 200 nM () were used. Error bars represent standard deviations on triplicate data.

**Figure 4.** The antimicrobial activity of salivaricin P (A) before and (B) after a 3 hour trypsin digestion reaction, against *Listeria innocua* DPC3572. (A) The top left well contains Sln1 alone, the top right well contains Sln2 alone and the bottom well contains Sln1 and Sln2 combined in equal amounts. (B) The top left well contains Sln1 treated with trypsin, the top right well contains Sln2 treated with trypsin and the bottom well contains trypsin treated Sln1 and Sln2 combined in equal amounts.
Figure 5. Individual and combined antimicrobial activity of Sln1 and Sln2-5. In each panel the top left well contains Sln1 alone, the top right well contains Sln2-5 alone (both at 50 µM concentrations) and the bottom well contains Sln1 and Sln2-5 combined in equal amounts against (A) E. faecalis DPC1142, (B) Lb. bulgaricus LMG 6901 and (C) L. innocua DPC3572.

Figure 6. Effect of (A) wild-type salivaricin P at concentrations of 0 nM to 250 nM and (B) trypsin resistant Sln1-5 and Sln2-3 variants at concentrations of 0 nM to 500 nM against Listeria monocytogenes NCTC11994.
Table 1. Sequences and MIC$_{50}$ of the salivaricin P component peptides and their variants against *L. innocua* DPC3572

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence$^a$</th>
<th>Length (aa)</th>
<th>MIC$_{50}$$^b$ (nM)</th>
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<tr>
<td>Sln1</td>
<td>K R GPNCVGNFLGGFLAGAAAGVPPLGPAIVGGANLGMVGGALTCL</td>
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<td>50</td>
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<td>Sln1-1</td>
<td>R GPNCVGNFLGGFLAGAAAGVPPLGPAIVGGANLGMVGGALTCL</td>
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<td>Sln1-2</td>
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<td>200</td>
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<td>100</td>
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<tr>
<td>Sln1-4</td>
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<td>Sln1-6</td>
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<td>RP WHVCAGIVGGGALIGAIGGPWSAVAGGISGGFASCH</td>
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<td>250</td>
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$^a$The carboxyl side of lysine (K) and arginine (R) represent trypsin specific cleavage sites.

$^b$MIC$_{50}$ of bacteriocin variant combined with wild type complementary peptide.
Table 2. Impact of tryptic digestion of Sln1 and Sln2 and their respective variants as determined by mass spectrometry

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Molecular Mass (Da)</th>
<th>Mass (Da) following 3 hr trypsin digest</th>
<th>Trypsin sensitivity ( ^a )</th>
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<tr>
<td>Sln1</td>
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<td>Sln1-5</td>
<td>4,136</td>
<td>4,136</td>
<td>R</td>
</tr>
<tr>
<td>Sln1-6</td>
<td>4,078</td>
<td>3,812</td>
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<tr>
<td>Sln2</td>
<td>4,284</td>
<td>880, 3,293</td>
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<tr>
<td>Sln2-1</td>
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<td>Sln2-2</td>
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<td>Sln2-3</td>
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<td>Sln2-4</td>
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<td>880</td>
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<td>Sln2-5</td>
<td>3,547</td>
<td>3,547</td>
<td>R</td>
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\( ^a \) S, sensitive; R, resistant.
Figure 1.
550  Sln1  KRGPNCVGNFLGGLFAAGAAAGVPLGPAGIVGGANLGVMVGALTCL
551  Abp118α  KRGPNCVGNFLGGLFAAGAAAGVPLGPAGIVGGANLGVMVGALTCL
552  Salα  KRGPNCVGNFLGGLFAAGAAAGVPLGPAGIVGGANLGVMVGALTCL
553  ****************************************
554  *****
555  Sln2  KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFASC
556  Abp118β  KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
557  Sal β  KNGYGGSGNRWVHCGAGIVGGALIGAIGGPWSAVAGGISGGFTSCR
558  ******************************************  **
559  Figure 2.
Figure 3.
Figure 4.
### Indicator strain

<p>| | |</p>
<table>
<thead>
<tr>
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<tr>
<td>(A)</td>
<td><em>Enterococcus faecalis</em> DPC1142</td>
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<tr>
<td>(B)</td>
<td><em>Lactobacillus bulgaricus</em> LMG 6901</td>
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<tr>
<td>(C)</td>
<td><em>Listeria innocua</em> DPC3572</td>
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**Figure 5.**
Figure 6.