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Purification and Characterisation of Nisin P Produced by a Strain of *Streptococcus gallolyticus*

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Abstract

Introduction: Against the backdrop of increasing resistance to conventional antibiotics, bacteriocins represent an attractive alternative, given their potent activity, novel modes of action and perceived lack of issues with resistance.
Aim: In this study, the nature of the antibacterial activity of a clinical isolate of *Streptococcus gallolyticus* was investigated.
Methods: Optimisation of the production of an inhibitor from strain AB39 was performed using different broth media and supplements. Purification was carried out using size exclusion, ion exchange and high-pressure liquid chromatography (HPLC). Gel diffusion agar overlay, MS/MS, *de-novo* peptide sequencing and genome mining were utilised in a proteogenomic approach to facilitate identification of the genetic basis for production of the inhibitor.
Results: Strain AB39 was identified as *Streptococcus gallolyticus* subsp *pasteurianus* and the successful production and purification of the AB39 peptide, named nisin P, with a mass of 3,133.78 Da, was achieved using BHI broth with 10% serum. Nisin P showed anti-bacterial activity towards clinical isolates of drug resistant bacteria, including MRSA, VRE and penicillin resistant *Streptococcus pneumoniae*. In addition, the peptide exhibited significant stability towards high temperature, wide pH and certain proteolytic enzymes and displayed very low toxicity towards sheep red blood cells and Vero cells.
Conclusion: To the best of our knowledge, this is the first production, purification and characterisation of nisin P. Further study of nisin P may reveal its potential for treating or preventing infections caused by antibiotic resistant Gram positive bacteria, or those evading vaccination regimens.

Introduction

Antimicrobial resistance (AMR) is widely stated by various international authorities and agencies as one of the leading global threats to human health. Many measures are required for controlling the AMR crisis, including searching for novel antibiotic drugs that potentially work with a new mode of action (1-6). The majority of antibiotics in clinical use were discovered being naturally produced by environmental microorganisms (7, 8), which could serve as the source of alternative antimicrobial approaches. Bacteriocins are gaining interest as one such alternative approach, due to a number of favourable properties, although a lack of research has been conducted compared with that carried out on conventional antibiotics (1, 9).

Bacteriocins are naturally produced ''ribosomally synthesised'' toxins found in many strains of bacteria and certain Archaea. They have activity towards other multidrug resistant pathogenic bacteria have and been considered as promising candidates for replacing traditional antibiotics (10-14). Approximately 99% of all bacterial strains are capable of producing at least one bacteriocin (15) however, without optimisation, most bacteria will not express bacteriocins under normal laboratory conditions (16, 17). Bacteriocins either exhibit a narrow spectrum of activity towards strains closely-related to the producer or a border spectrum of activity by targeting various species of Gram-positive and negative bacteria, viruses, fungi, parasites or even tumour cells (13, 18-20). Due to the safe long utilise of certain bacteriocins and their producing bacterial ''probiotic'' strains, particularly lactic acid producing bacteria (LAB) and their anti-bacterial products (e.g nisin-A), they have been considered as Generally Regarded as Safe (GRAS) and their producers possess Qualified Presumption of Safety (QPS) status (21). Due to the fact that bacteriocins and their producing bacteria possess certain coveted features, such as broad

spectrum, high potency, stability to pyrolytic enzymes and limited toxicity towards targeted hosts, they have become attractive candidates and are the focus of much research in human and animal health, food preservation and agriculture (17, 21-26).

Gram positive pathogens are still a major problem in human health, particularly drug resistant strains (27), emphasising the need for the discovery of novel biological agents targeting this group of bacteria. This study focuses on a strain of *Streptococcus gallolyticus*, a species originally described as *Streptococcus bovis*. A small number of bacteriocins have been identified from various strains *S. bovis* of this bacterium, including Bovicin HC5, Bovicin 255, Bovicin HJ50 and macedocin ST91KM (28-33).

In this project, and to the best of our knowledge, this is the first report of the production, physicochemical characterisation and evaluation of toxicity of a nisin related antibacterial inhibitor (nisin P), produced by *S. gallolyticus* subsp *pasteurianus*, formerly known as *S. bovis* biotype II/2 (34). Nisin P exhibits a spectrum of activity towards drug resistant clinically relevant Gram positive pathogenic bacteria including MRSA, VRE and drug resistant *S. pneumoniae* with very limited toxicity towards sheep erythrocytes and monkey kidney epithelial cells [Vero cells]. Interestingly, nisin P did not show activity towards breast adenocarcinoma [MCF_7] and liver hepatocellular carcinoma [HepG2] cells, although other nisin variants (nisin A & nisin Z) have been reported for their activity towards these malignancies (35-39).

Materials and Methods

Collection of isolates and antimicrobial screening

Strain AB39 was identified in a collection of bacterial strains that had been co-isolated with *Escherichia coli* from suspected urinary tract infections. Samples were obtained at the University of Plymouth NHS Trust Hospital, Plymouth, UK in 2016. Antimicrobial activity was revealed by screening against *Micrococcus luteus* [Strain-1.1-University of Plymouth (UoP)] *Staph*. *aureus* (NCTC 12981), *S. pneumoniae* (NCTC 12695), *Escherichia coli* (DH5α) and *Klebsiella pneumoniae* (NCTC 9633). Initial detection of antagonistic strains was obtained *via* simultaneous antagonistic assays (40). Columbia Blood agar (CBA) plates (Oxoid) were used for culturing bacteria in an aerobic atmosphere supplemented with 5% CO_2 at 37°C. Strain AB39 was chosen for detailed investigation due to the absence of haemolytic activity on CBA and activity toward drug resistant clinical strains. A spot-on-lawn assay with certain modifications (41) and well diffusion assays (42) were used to track active fractions of the peptide during various steps of optimisation and purification. *M. luteus* bacterium was used throughout all steps of the purification and characterisation.

Optimising bacteriocin production

An extensive range of broth media, growth conditions and incubation times were utilised to obtain the highest yield of the produced peptide. To determine the best medium and growth conditions leading to high yield of the peptide, a 2-fold dilution was carried out using culture supernatants from each medium and growth condition used. This was subjected to a spot-on-lawn assay (41) and all observed inhibitory zones were measured using the following formula: AU/ml = (reciprocal of the highest active dilution) / (volume of the sample) X 1000 (43). This experiment was carried out in triplicate.

Purification of the antibacterial peptide

S. gallolyticus grown in BHI (Oxoid) plus 10% serum (Gibco™ Life Technologies, UK) was centrifuged at 7400 *g* for 20-30 minutes and the supernatant was subjected to preliminary purification using Strata® C18-E columns (Phenomenex® Ltd., Macclesfield, UK). Fractions of the AB39 inhibitor were eluted using acidified (pH2) methanol (MeOH; Fisher Scientific, Loughborough, UK) at 30%, 50% 70%, 80% and 90% concentrations. MeOH was then evaporated from all active fractions using a rotary evaporator (LABOROTA (4001) Heidolph, Schwabach, Germany), which were then combined and run into a C18 Sep-Pak® Plus column (Waters Corporation, Milford, Massachusetts, USA) with 2.5% increments of Acetonitrile [(ACN) Fisher-Scientific] used for elution of AB39 peptide. ACN from active fractions was evaporated using the Biotage® V-10 Touch evaporation system (Biotage, Uppsala Sweden), followed by Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) [250 × 10 mm C18 column (Agilent, Edinburgh, UK)] using a Gilson HPLC purification system (Gilson, Dunstable, UK). Fractions were collected *via* an automated collector and a gradient of 10-90% ACN at a flow rate of 0.5 column volumes (CV) per minute. An activity-based tracking system *via* the spot assay was applied during all steps of purification. Purified nisin P was tested against various species of Gram positive and -negative bacteria.

SDS-PAGE and gel diffusion agar overlay

Purified fractions of the AB39 peptide were run into an SDS-PAGE gel (Bolt™ Mini Gels; Invitrogen™, Life Technologies, Warrington, UK) following the manufacture's instructions. Aliquots (10 µl) of a pre-stained protein standard (See Blue® Plus2; Life Technologies) and the prepared sample were loaded into designated wells and the gel run at 165 volts for 32 minutes followed by staining with Instant Blue stain (Expedeon Ltd, Cambridge, UK) for 30-60 minutes. SDS-gels were then washed multiple times with ultra-pure water, minimally for 4 hours. Individual lanes were excised as strips and placed aseptically onto a Nutrient Agar (NA) plate. Molten sterile NA was seeded at ~45°C with *M. luteus* at a suspension equivalent to a 0.5 McFarland standard that had been diluted to 1:100 (44). This was poured over the gel strip and incubated aerobically at 37°C overnight followed by observation of any zones of inhibition in the lawn.

Liquid chromatography–mass spectrometry (LCMS) and *de-novo* peptide sequencing

Filter Aided Sample Preparation (FASP) was conducted on active fractions from HPLC, followed by digestion using LysC (45). The digested sample was then cleaned and desalted *via* the StageTip technique (45) and concentrated samples were transferred into glass micro-vials ready for MS/MS analysis. Peptides were separated on a Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberly UK). A 3 µl of sample was loaded in 0.1% trifluoroacetic acid (TFA) and acetonitrile (2% acetonitrile in 0.1% TFA) onto an Acclaim Pep Map100 µm × 2 cm, 3 µm C18 nano trap column, at a flow rate of 5 µl/min, bypassing the analytical column. Elution of bound peptides was performed with the trap column inline with an Acclaim PepMap C18 nano column 75 µm × 25 cm, 3 µm, 100 Å (Analytical Column) with a linear gradient of 96% buffer A and 4% buffer B to 60% buffer A and 40% buffer B, (Buffer A: 0.5% Acetic Acid, Buffer B: 80% acetonitrile in 0.5% acetic acid) at a constant flow rate of 300nl/min over 60 minutes. The sample was ionized in positive ion mode using a Proxeon nano spray ESI source (Thermo Fisher Hemel UK) and analyzed in an Orbitrap Velos Pro FTMS (Thermo Finnigan, Bremen, Germany). The Orbitrap Velos Pro instrument underXcalibur2.1 software was operated in the data dependent mode to automatically switch between MS and MS/MS acquisition. MS spectra of intact peptides (m/z 350-1600) with an automated gain control accumulation target value of 1000000 ions were acquired with a resolution of 60000. The ten most intense ions were sequentially isolated and fragmented in the linear ion trap by collision induced dissociation (CID) at a target value of 10,000 or maximum ion time of 200 ms. A dynamic

190 exclusion of ions previously sequenced within 45" was applied. All the singly charged and
191 unassigned charge state ions were excluded from sequencing. Typical mass spectrometric
192 conditions were: spray voltage, 2.3 kV; no sheath and auxiliary gas flow; heated capillary
193 temperature, 275˚C; normalized CID collision energy 30% for MS2 in LTQ. The ion
194 selection threshold was 10000 counts for MS2. An activation q = 0.25 and activation time
195 of 30 ms were used (46). Raw data from the MS analysis was analysed using the
196 streptococcal protein/peptide database (www.uniprot.org). In addition, *de-novo* peptide
197 sequencing was performed on the MS/MS data for the AB39 peptide. All sequence tags
198 obtained were compared to the producer strain draft genome using PEAKS Studio 7
199 software (Bioinformatics Solutions, Waterloo, Canada) (47).

200 **Genomic DNA extraction and sequencing**

201 The genomic DNA of strain AB39 was extracted according to the instructions of the
202 DNAasy Blood and Tissue Kit (Qiagen, UK). Sequencing reads were obtained by
203 MicrobesNG, Birmingham University, UK (https://microbesng.uk/) using the Illumina HiSeq
204 platform (Illumina Inc, USA). Reads were trimmed using Trimmomatic (48) and their
205 quality was evaluated using local scripts (Birmingham University, UK) in combination with
206 multiple software tools, including SAMtools (49), Bedtools (50) and BWA-MEM (51).
207 Assembly was accomplished by using the SPAdes *de-novo* assembly tool (52) with
208 calculated quality determined *via* QUAST (53). Annotation and taxonomic identification
209 were accompanied by using PROKKA prokaryotic (54) and Kraken (55) tools, respectively.

210 **Genome mining**

211 The draft genome was analysed *in-silico* using BAGEL-3 (56) and antiSMASH-4.0 (57).
212 For homology searches, amino acid sequences of all *in-silico* predicted peptides and/or
213 their biosynthetic gene clusters (BGCs) were searched against relevant protein/peptide
214 databases, including UniProt (57), BACTIBASE [http://bactibase.hammamilab.org] (58, 59),
215 ProtBLAST/PSI-BLAST and the NCBI using the Basic Local Alignment Search Tool
216 (BLAST) [https://blast.ncbi.nlm.nih.gov/Blast.cgi]. Amino acid sequences of *in-silico*
217 predicted peptides were aligned with other similar/relevant peptides using the Jalview
218 software multiple sequence alignment editor (60). Based on the percentage of amino acid
219 identity, neighbour-joining trees were drawn using Jalview software to facilitate the
220 determination of peptide relatedness. Moreover, whole putative BGCs were manually
221 aligned with previously discovered ones, or using the genome comparison visualizer
222 Easyfig (61). Sequences for the coding regions of genes in the nisin P cluster have been
223 deposited to ENA under the accession numbers MN449418 to MN449428.

224 **Proteogenomic analysis and primary structure identification of nisin P**

225 Predicted masses, obtained from the purified active fractions *via* SDS-PAGE gel diffusion
226 agar overlay and MS/MS fragmentation, were linked with bioinformatically obtained data in
227 order to identify the genetic basis for production of the antibacterial activity produced by
228 strain AP39. The primary structure of mature amino acid sequences of peptides was
229 determined using the PepDraw tool [http://pepdraw.com/] to investigate the relatedness of
230 NPs at the level of their primary structure.

231 **Stability towards hydrolytic enzymes, heat and pH**

232 Triplicate samples of purified AB39 peptide were treated with 1 mg/ml of α-amylase, α-
233 chymotrypsin, lipase, protease and trypsin (Sigma-Aldrich), whereas 10 mg/ml of
234 proteinase K (Sigma-Aldrich) was used. All treated samples were serially diluted and all
235 dilutions were assayed for their activity against *M. luteus* using the agar well diffusion
236 assay. Based on the activity of the non-treated control sample, the percentage of retained
237 activity was calculated.

238 For heat stability, triplicate samples (~200 µl aliquots) of AB39 inhibitor were heated at
239 80°C for 1-4 hours using a heating block or subjected to autoclaving at 121°C for 15
240 minutes. In addition, AB39 peptide was incubated at a wide range of pH (3-8). The activity
241 of treated samples was evaluated using the spot-on-lawn assay.

242 **Haemolytic activity**

243 The haemolytic activity of purified of nisin P towards sheep RBCs was assessed as
244 formerly described (62) using AB39 peptide (1:10) serially diluted in PBS. PBS and 1%
245 Triton™-100X (Sigma-Aldrich) were utilised as positive and negative controls, respectively.
246 Haemolysis was assessed following incubation at 37°C for 60 minutes, after which time,
247 samples were centrifuged at 2000 rpm for 10 minutes, and absorbance of the
248 supernatants was measured at 560nm using a plate reader (BMG Labtech Ltd, Aylesbury,
249 UK). The overall percentage haemolysis of the peptide was calculated using the following
250 formula: % haemolysis = 100 (Absorbance of treated sample with the peptide –
251 Absorbance of untreated samples) / (Absorbance of treated sample with Triton-X –
252 Absorbance of untreated samples) (63).

253 **Toxicity study using selected cell lines**

254 Toxicity of the AB39 peptide was assessed towards Vero (Catalogue No. 85011422),
255 MCF_7 (Catalogue No. 86012803) and HepG2 (Catalogue No. 85011430) cell lines, which
256 were purchased from the European Collection of Authenticated Cell Cultures (ECACC)
257 (Public Health England, Porton Down Salisbury, UK). Based on previous methods (39),
258 nisin A [2.5% balance sodium chloride (Sigma-Aldrich)] was prepared and used as a
259 positive control i due to its reported anticancer activity. Cells were recovered and grown
260 according to the supplier's guidelines, and growing cells (passage No: 8-16) were
261 trypsinised before culture in 96 well tissue-culture plates (Grenier, Gloucestershire, UK)
262 using phenol red free culture media, 1 X 10^{-4} cells/well seeding density in a volume of 90
263 µl/well and using overnight incubation at 37°C with 5% CO_2. Experiments were conducted
264 using 10 µl/well from each prepared stock of concentrations of nisin A, AB39 compound
265 and negative controls (10 µl of diluent plus 90 µl of each cell culture medium) with
266 incubation at 37°C plus 5% CO_2 for 96 hrs. Sodium phosphate (5mM; pH 2) was used as a
267 diluent for nisin A (39), whereas the lyophilised AB39 peptide was dissolved in 0.01%
268 acetic acid plus 0.2% Bovine serum albumin (BSA). Cell viability assay was assessed
269 using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS)
270 (Promega, Southampton, UK) according to the manufacturer's instructions with
271 absorbance measured at 490nm. Obtained readings were analysed using the GraphPad
272 Prism software (6.0) (64) and the IC_{50} of compounds towards cell lines was calculated.
273 Cytopathic effects (CPEs) of treated and untreated cells were monitored using the 40X
274 lens of an invented microscope and a digital microscopic camera (DCM-5-10, 5.0M pixels).

275

276 **Results**

277 **Optimising the production and purification of the AB39 inhibitor**

278 A high yield of the inhibitor produced by strain AB39 (1600 AU/ml) was only achieved in
279 BHI plus 10% FBS that was incubated statically and aerobically with 5 % CO_2 for 6-10
280 hours at 37°C. Extraction of the AB39 inhibitor was achieved using three successive steps
281 of purification, in which the active fraction was eluted at 80% MeOH plus 0.01% TFA, 27.5%
282 MeOH plus 0.01% TFA and 45% ACN plus 0.01% TFA or 13-14 minutes retention time
283 (RT) *via* Strata ® C18-E column, Sep-Pak® C18 cartridge and RP-HPLC, respectively.

284 **SDS-PAGE and gel overlay diffusion assay**

285 Purified AB39 inhibitor was run into an SDS-gel, in which observed bands possessed
286 masses that ranged between ≥ 3 kDa and ≤ 7 kDa (Fig. 1). The gel diffusion agar overlay
287 assay resulted in a zone of inhibition caused by a band within the same range, although
288 the size of the zone appeared to be larger in the mass range between ≥ 3 kDa and ≤ 4
289 kDa (Fig. 1).

290 **MS/MS analysis and *de-novo* sequencing**

291 Fragments generated during MS/MS analysis of AB39 active fractions mapped to multiple
292 peptides not related to antimicrobial proteins. However, fragments that mapped to nisin U
293 peptide, which is produced by *S. uberis* (65), were the most predominant (MS/MS count of
294 66). In addition, *de-novo* peptide sequence analysis of the same fraction queried against
295 the draft genome sequence of the producer led to the identification of a nisin U-like peptide,
296 with 84% identity/coverage.

297 **Identification of the AB39 producing strain and genome mining for BCGs**

298 The AB39 producing strain was identified as *S. gallolyticus* (100% identity & coverage)
299 based on the 16 S rRNA gene sequence (data not shown). *In-silico* analysis revealed two
300 different putative bacteriocins. One of these showed 90.9% identity with nisin U (Fig. 2),
301 indicating that this was the peptide that had been purified from *S. gallolyticus* strain. The
302 other predicted peptide did not show any significant match to known antimicrobial peptides
303 (AMPs). The amino acid sequence of the AB39 peptide aligned with all of published nisin
304 variants and a neighbouring joining tree was generated (Fig. 3).

305 Only when an extensive search in the literature was performed was it revealed that a
306 previously described peptide had 100% identity with the AB39 purified peptide. During an
307 *in-silico* study of the genome of a strain of *S. gallolyticus*, brief mention was made of a
308 peptide designated nisin P, based on similarity to other nisin variants (66). As nisin P
309 showed 100% identity with the AB39 peptide, the AB39 peptide has been called nisin P.
310 Although the sequence has been previously reported, this is the first study to produce,
311 purify and characterise the nisin P.

312 **Genetic organisation and annotation of the nisin P locus**

313 Based on the fact that genes required for the biosynthesis of potential bacteriocins are
314 usually organised in close proximity to each other (67), genes upstream and downstream
315 of the AB39 structural gene were manually annotated against relevant databases. This
316 analysis led to the determination of the genetic organisation of all required genes (total n=
317 11), with an indication of their potential function and the overall identity of each gene
318 compared with the closest reported variant of this peptide (nisin U) (65) (Fig. 4 & Table 1).
319 This comparison revealed synteny in the operons, but with a possible small inversion in
320 the AB39 genome downstream of the structural gene, but in the non-coding region.

321 **Primary structure of nisin P, nisin A and nisin Z**

322 With respect to the relatedness/similarity between nisin A, nisin Z, nisin P and nisin U at
323 the level of the primary structure of their mature sequences, it is apparent that nisin A and
324 nisin Z share almost identical structures, as do nisin U and nisin P (Fig. 5). There are
325 subtle differences between the structures of these two pairs of peptides.

326 **Physiochemical properties of nisin P**

327 Stability of nisin P towards hydrolytic enzymes [protease, trypsin, α-chymotrypsin, α-
328 amylase and lipase], pH and high temperature was determined. Based on the antimicrobial
329 activity of a non-treated sample of nisin P (control) [2560 AU/ml] towards *M. luteus*, the
330 activity of samples treated with protease, trypsin or α-chymotrypsin was reduced by about

75%, while only 12.5% activity was retained in either α-amylase or lipase treated samples. In addition, although the exact percentage of retained activity of nisin P was not calculated, its activity was detected in a wide range of pH (3-8 pH) and temperatures [4-8°C for 4 weeks, 80°C for 30 minutes and after autoclaving (121°C, 15 psi and for 15 minutes)].

Spectrum of activity

Nisin P showed antibacterial activity towards various type strains and clinical isolates of Gram positive drug resistant bacteria, whereas the tested species of *Listeria* and *Bacillus*, strains of *Streptococcus agalactiae* and *Streptococcus anginosus* and all Gram-negative species were not inhibited by nisin P (Table 2).

Haemolytic activity towards sheep RBCs

A double dilution of the highest concentration of nisin P obtained for active fractions, was the highest concentration of peptide able to be tested and this did not show any significant toxicity towards sheep enterocytes (0.32% lysis).

Toxicity towards eukaryotic cell lines

Nisin A showed significant activity towards MCF_7 and HepG2 cells, and slightly reduced toxicity towards Vero cells (Fig. 6). Therefore, the IC_{50} of nisin A is as follows: 362µg/ml, 451µg/ml and 623µg/ml for MCF_7, HepG2 and Vero cells, respectively. In contrast, nisin P failed to show any significant activity towards these cell lines and the highest concentrations tested led to 15%, 5% and 20% reduction of MCF_7, HepG2 and Vero cell viability, respectively. Thus, it was not possible to determine the IC_{50} of nisin P towards these cells. Furthermore, all cells treated cells with nisin A showed obvious CPEs, including cell rounding, shrinking, detachment and/or vacuoles indicting their death, whereas nisin P treated cells, especially MCF_7 and HepG2, did not show any obvious CPE and appeared to be healthy cells compared to their controls. Nevertheless, there was a slight CPE (cell rounding) observed in Vero cells treated with nisin P (Fig. 7).

Discussion

Due to the growing worldwide crisis of AMR (3) as well as the low rate of discovery of new antibiotics, worldwide human health is under a serious threat (2, 68, 69). Thus, urgent action is needed to tackle the global crisis of AMR, including discovery of new antibacterial agents with unique modes of action. Various candidates exist, including the bacteriocins (3, 14, 70-72). Certain bacteriocins (e.g nisin) possess a dual mode of action towards sensitive bacteria and this might explain the limited tendency for development of resistance to this class of AMPs (73, 74). The aim of this study was to characterise the inhibitor produced by a bacterium that was initially identified during a screen of various environmental and clinical bacterial strains. The focus of the active agent was determined towards a range of multidrug resistant (MDR) bacteria including MRSA, VRE and drug resistant *S. pneumoniae*. Here we describe the production, purification, physicochemical properties, genetic organisation and the toxicity of nisin P peptide, which has only been identified *in silico* in previous studies.

A high yield of nisin P was obtained from *S. gallolyticus* between 6-10 hours, which correlated well with the time of incubation for the high level production of nisin A from *Lactococcus lactis* (75). Reduced activity of both peptides after 10 hours of incubation might be due to a reduction in the density of the producing cells, adsorption of the produced compound to the producing cells or potential proteolytic degradation (75, 76).

SDS-gel analysis can be a useful tool for predicting the MW and purity level of a produced peptide (77). Therefore, the purest fraction containing nisin P was run into an SDS- gel and the MW of the observed bands fell within the range of the calculated theoretical MW of nisin P (3.133 KDa). In addition, the inhibitory zone that was observed towards *M. luteus* using the gel diffusion overlay assay corresponded to bands within the same MW range. Furthermore, MS/MS analysis of the AB39 active fraction from HPLC generated multiple hits (total n=114) in the UniProt database, but the hit with the highest MS/MS count (n=66) mapped to nisin U (65). These findings support the suggestion that the purified AB39 compound is closely related to nisin U.

Bioinformatic analysis that was conducted on the draft genome of *S. gallolyticus*, revealed nisin P and all required genes for its biosynthesis. BGCs encoding bacteriocins should encompass all required genes for the biosynthesis and production of inhibitors (e.g self-immunity, regulation and exportation), and these are usually found in close proximity to the structural gene (78, 79). Genes of the nisin P cluster were syntenic with those within the nisin U cluster with 94.6% and 82.1% maximum and minimum identity, respectively. The organisation of these clusters is unlike that of the genes encoding nisin A (80). Based on overall homology and relatedness of the AB39 inhibitor (nisin P) to sequences for other nisin variants including nisin A (81), nisin Z (82), nisin Q (83), nisin U (65), nisinF (84), nisin H (80) and nisin O (85), it appeared to be a new variant of nisin U.

The AB39 peptide showed 100% identity to a putative nisin variant predicted from the genome of a strain of *S. gallolyticus*, which was called nisin P (66). Therefore, the AB39 peptide has been named nisin P, to avoid any potential ambiguity. In addition, in the study by Zhang and colleagues (65), only the structural gene was reported and here the entire operon has been characterised. Furthermore, in our study, optimised laboratory production, physicochemical analysis, antibacterial and anticancer activity determination and toxicity testing of nisin P have been conducted, for the first time.

The antimicrobial activity of nisin P was reduced by 75% following treatment with protease, trypsin or α-chymotrypsin. This finding, as well the availability of three potential trypsin cleavage sites within nisin P sequence confirm its proteinaceous nature (18, 21, 22). Additionally, nisin P was found to be more sensitive to α-amylase and lipase enzymes leading to about 12.5% retained activity and this might suggest the availability of polysaccharide and lipid moieties within nisin P (41). Furthermore, although the percentage of retained activity of nisin P was not calculated, its activity was detected at a wide range of pH (3-8 pH) and temperatures. The observed stability of nisin P under these conditions correlates with that reported for nisin A (86), emphasising their potential effectiveness for use in various environments.

Nisin P displayed very limited levels of toxicity towards sheep RBCs at the highest concentration tested. Despite the fact that nisin A is approved by the USA FDA as a food preservative and it has been used globally for more than five decades (75, 80), further *in vitro* and *in vivo* evaluation of nisin P toxicity is required. Nevertheless, the low levels of haemolysis might support speculation about a promising potential for use of nisin P to treat bacterial infections caused by drug resistant bacteria in humans and/or animals.

Nisin P exhibited biological activity towards various genera of Gram positive bacteria, including species of *Micrococcus*, *Streptococcus*, *Staphylococcus* and *Clostridium*. However, nisin P failed to inhibit the growth of all tested species of *Bacillus* and *Listeria*, and strains of *S. anginosus* and *S. agalactiae*. It was not active against Gram-negative bacteria. Nisin A is known for its activity towards species of *Bacillus, Listeria, Streptococcus* and *Staphylococcus* (75). The nature of the resistance of some streptococcal indicators to nisin P is yet to be explained. Nevertheless, based on the

reported biological activity towards bacterial species, nisin A and nisin Z are found to be the most effective forms of nisin to date, and, thus, more limited activity from other variants would be expected, especially in *Streptococcal* derived nisins (e.g nisin U) (80, 86). This might be due to the increased variations within the amino acid sequences of Streptococcal nisins (nisin U, nisin H and nisin P) compared to lactococcal nisins (nisin A, nisin Z, nisin F, nisin Q) and the increase in the variation might be claimed to be inversely linked with biological activity (65, 80).

Nisin A and nisin Z have been claimed to be the most effective forms of nisin variants and their activity towards cancer cells including MCF_7 and HepG2 has been described (36, 37, 39, 86). Therefore, toxicity of nisin P towards Vero, MCF_7 and HepG2 cells was evaluated to reveal whether or not selective activity towards cancer cells exists. Nisin A showed significant toxicity, which was defined by at least 50% reduction of viable cells, towards cancer and Vero cells. The toxicity of nisin A appeared to be less towards Vero cells than MCF_7 and HepG2 cells, as has been described previously (39) and this might indicate its selectivity towards malignancies and its potential promise as a cancer therapeutic drug. In contrast, all tested concentrations of nisin P did not show any significant toxicity towards tumour and Vero cells. Images of Vero, MCF_7 and HepG2 cells treated with nisin A showed clear CPE, while there was either no or very little CPE in cells treated with nisin P. Nisin P was only marginally more toxic towards Vero cells than MCF_7 cells and HepG2 cells at 2500 µg /ml after 96 hours of exposure. However, differences in activity between cell lines was not statistically significant, indicating that nisin P does not possess any selective activity towards the particular cancer cells tested here. This might limit its potential use as a drug for treating tumour cells, though activity against additional cell lines could be tested. Nevertheless, due to the limited toxicity of nisin P towards Vero cells compared to nisin A, it still holds promise for potential use to treat bacterial infections caused by certain species within humans or/and animals. The variations within the amino acid sequences between nisin P and nisin A might be the only reason behind the distinct toxicity profile of nisin P towards bacterial, Vero, MCF_7 and HepG2 cells, which is unlike that described for nisin A (36, 37, 39, 80, 86). This is because the determined primary structure of nisin A and nisin Z are almost identical and distinct from that of nisin U and nisin P, which may give insight into experiments to engineer nisin to enhance antibacterial or anti-cancer activity.

Conclusion

The first successful production, purification and characterisation of the nisin P peptide has been achieved from a strain of *S. gallolyticus* subsp*. pasteurianus* in this study. Nisin P showed antibacterial activity towards MDR bacteria including MRSA, VRE and drug resistant *S. pneumoniae* with very limited toxicity towards sheep erythrocytes and eukaryotic cells [Vero cells]. Consequently, nisin P might hold a promise to treat or prevent bacterial infections caused by these organisms within humans and animals.

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472 **Conflict of interest declaration**

473 All authors have nothing to declare.

474

475 **References**

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Figure legends and table headings

Figure 1. Images of an SDS-gel ladder [A], SDS-gel [B] and gel diffusion overlay of the purified AB39 peptide [C]. Red dashed lines indicate the inhibitory zone caused by the peptide and its range of mass (3-7 kDa).

Figure 2. [A] Biosynthetic gene cluster (BCG) predicted by the BAGEL-3 mining tool from the draft genome of *S. gallolyticus* (AB39 strain), in which all genes required for the biosynthesis of the predicted peptide are present, as well as the structural gene (green). [B] The amino acid sequences of the putative peptide (AB39) showed 90.9% identity with nisin U that was described in *S. uberis* (65).

Figure 3. [A] Alignment the amino acid sequences of the AB39 peptide with all reported nisin variants [B] neighbouring joining tree generated for all aligned sequences to determine their relatedness.

Figure 4. Genetic organisation of all genes (total n=11) that are required for the biosynthesis of nisin P, and the amino acid sequences of all genes were aligned with those encoding the nisin U peptide using Easyfig. The homology is a colour gradient, in which 100% and 82% homology are indicated by the blue and red colours, respectively. **Note:** *nspP* = protease (cleavage) gene, *nspR* & *nspK* = regulation genes, *nspF*, *nspE*, *nspG* & *nspI* = immunity genes, *nspB* & *nspC* = genes involved in the post-translational modifications (PTMs) and *nspT* = transportation gene.

Figure 5. Primary structure of nisin A, nisin Z, nisin U and nisin P, as determined using the PepDraw tool [http://pepdraw.com/].

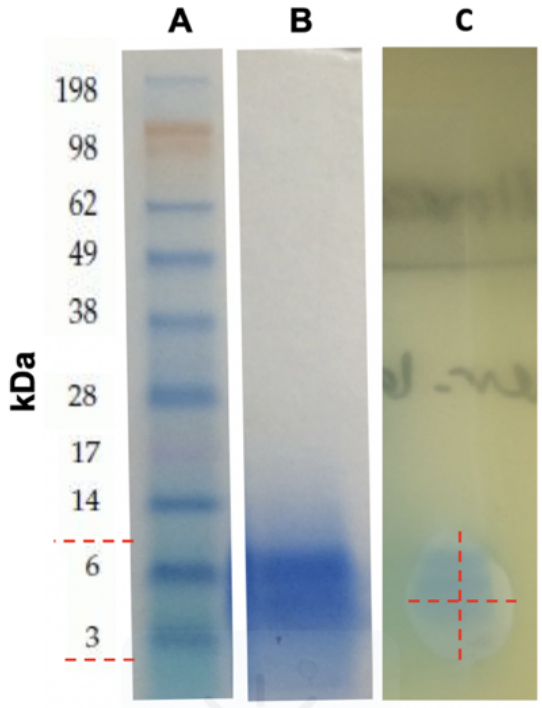
Figure 6. Toxicity of nisin P [A] and nisin A [B] towards breast adenocarcinoma (MCF_7), liver hepatocellular carcinoma (HepG2) and monkey kidney epithelial cells (Vero cells) using different concentrations of these peptides and 96 hours of exposure. 'n.s' = not statistically significant.

Figure 7. Images of treated eukaryotic cells [breast adenocarcinoma (MCF_7), liver hepatocellular carcinoma (HepG2) and monkey kidney epithelial cells (Vero cells)] with nisin A or nisin P (2500 µg/ml and 96 hours exposure) and non-treated cells (controls).

Table 1. Genes that are likely to be required for the biosynthesis of the AB39 peptide (nisin P) and their predicted functions, based on their synteny and homology with genes encoding the nisin U peptide (65).

Table 2. Spectrum of activity of purified nisin P peptide towards various Gram positive and -negative indicator strains. The inhibitory zones were measured in an arbitrary unit (AU), in which '+' is the smallest zone while '++++' was designated for the largest zone. **Note:** - = resistance of indicator/s, NCTC = National Collection of Type Cultures, UoP = University of Plymouth, VRE = Vancomycin-resistant *Enterococcus*, MRSA = Methicillin-resistant *Staphylococcus aureus*, MSSA = Methicillin-sensitive *Staphylococcus aureus* and PR = penicillin resistant.

749 Figure 1



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752 Figure 2

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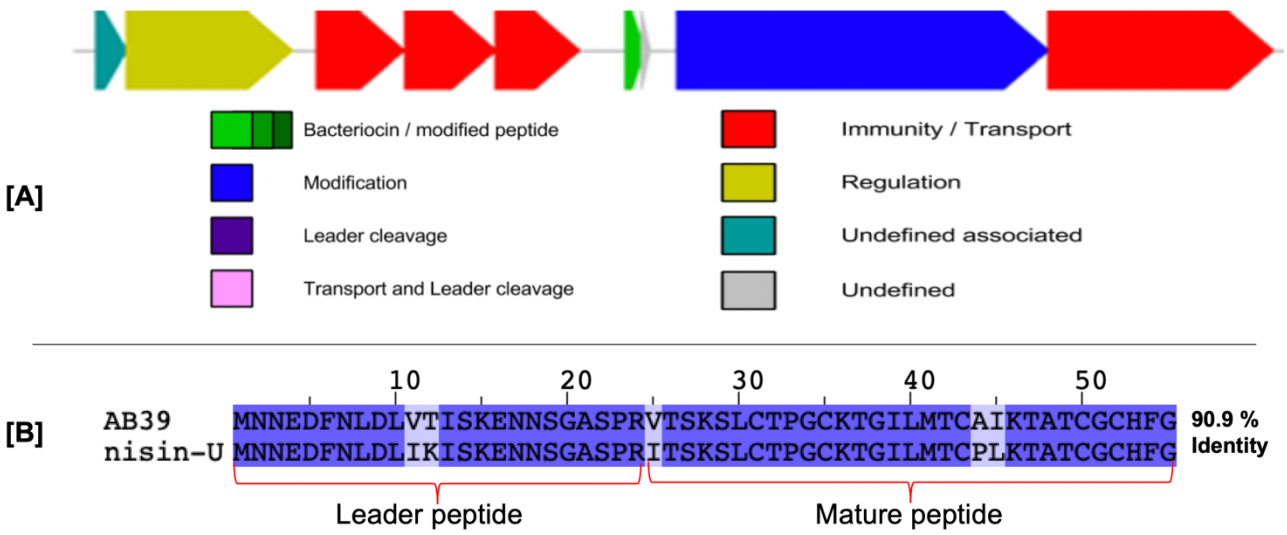

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Figure 3

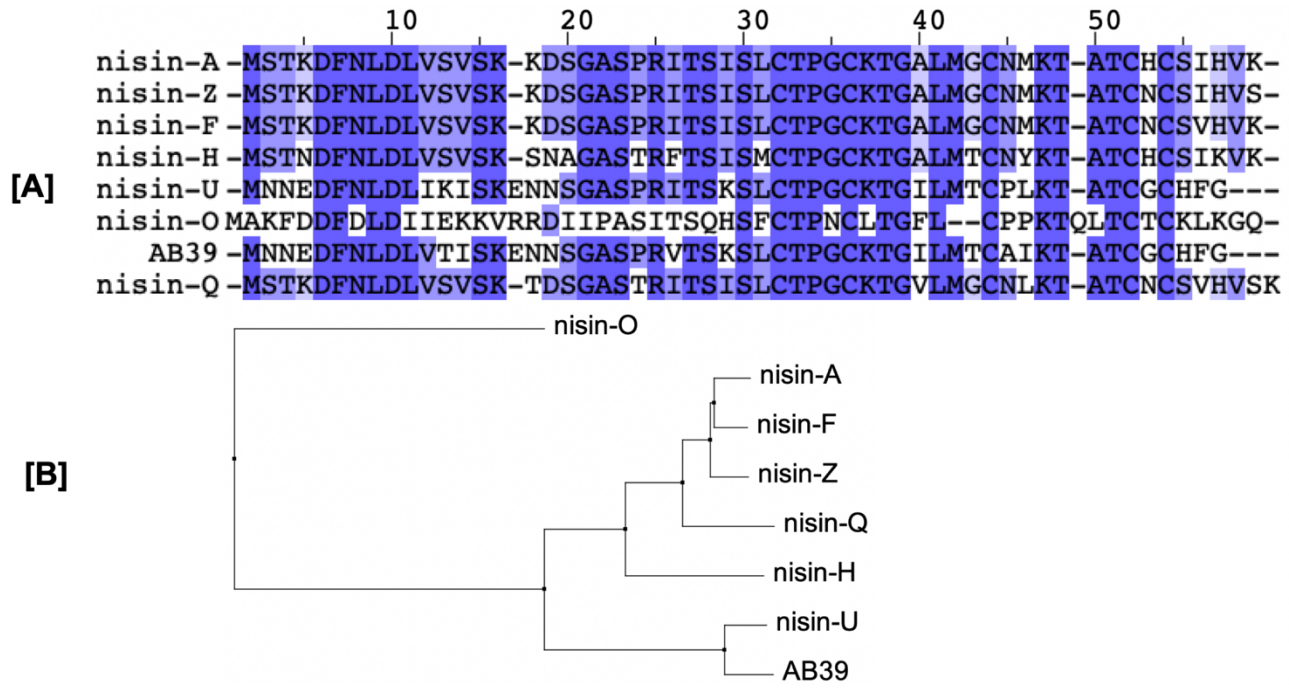
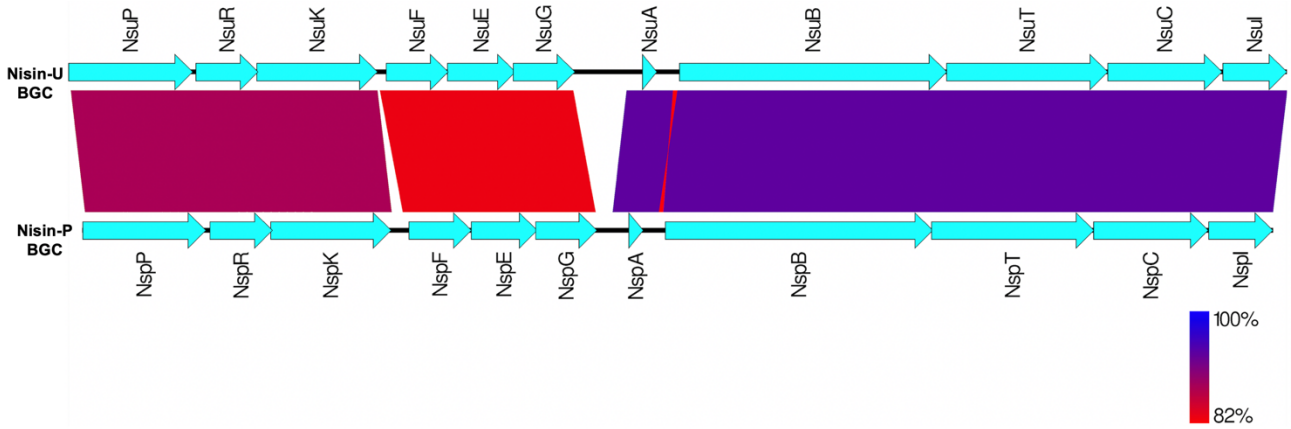


Figure 4



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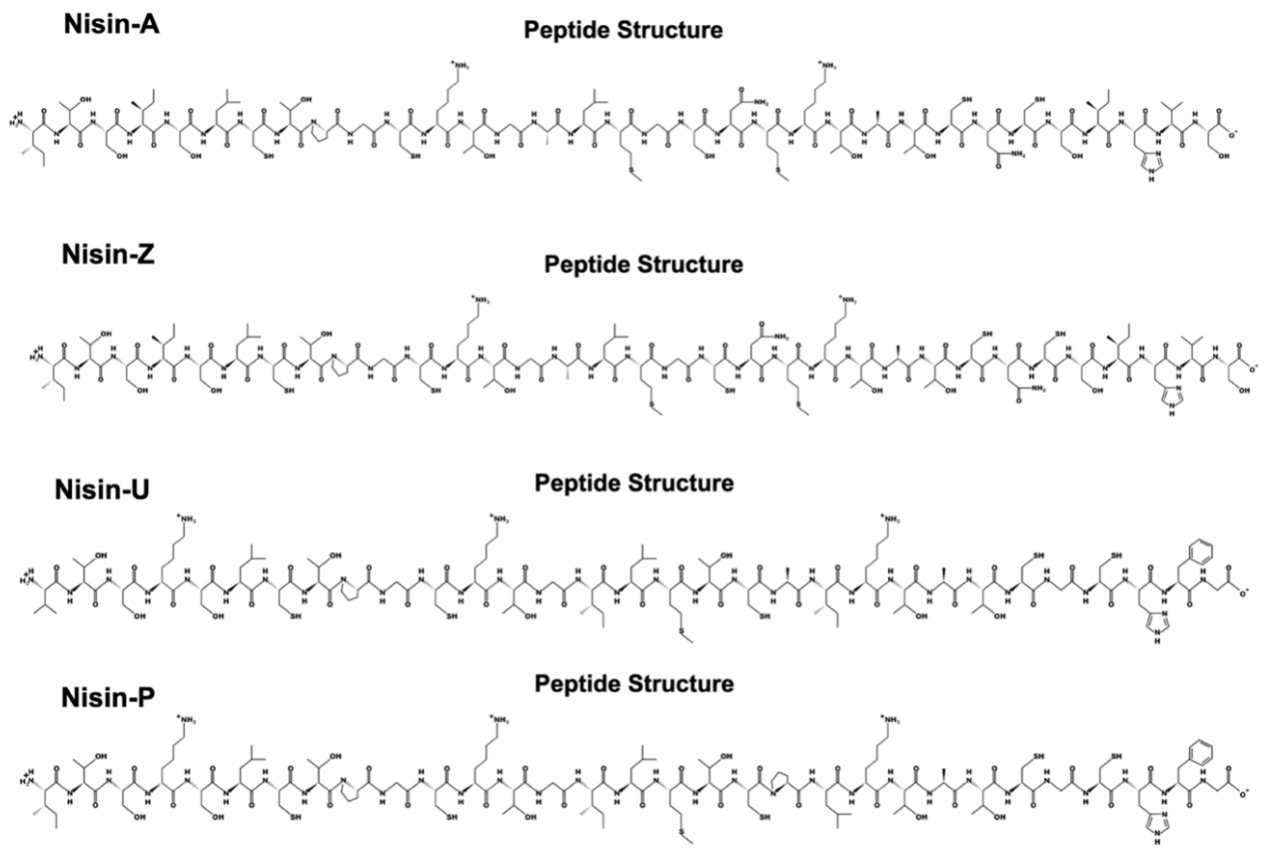

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769 Figure 6

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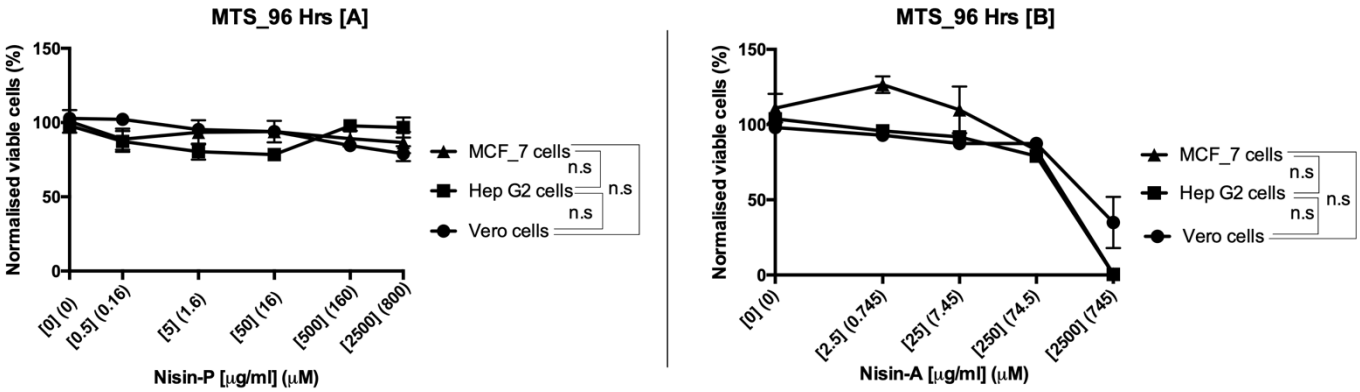

771 Figure 7

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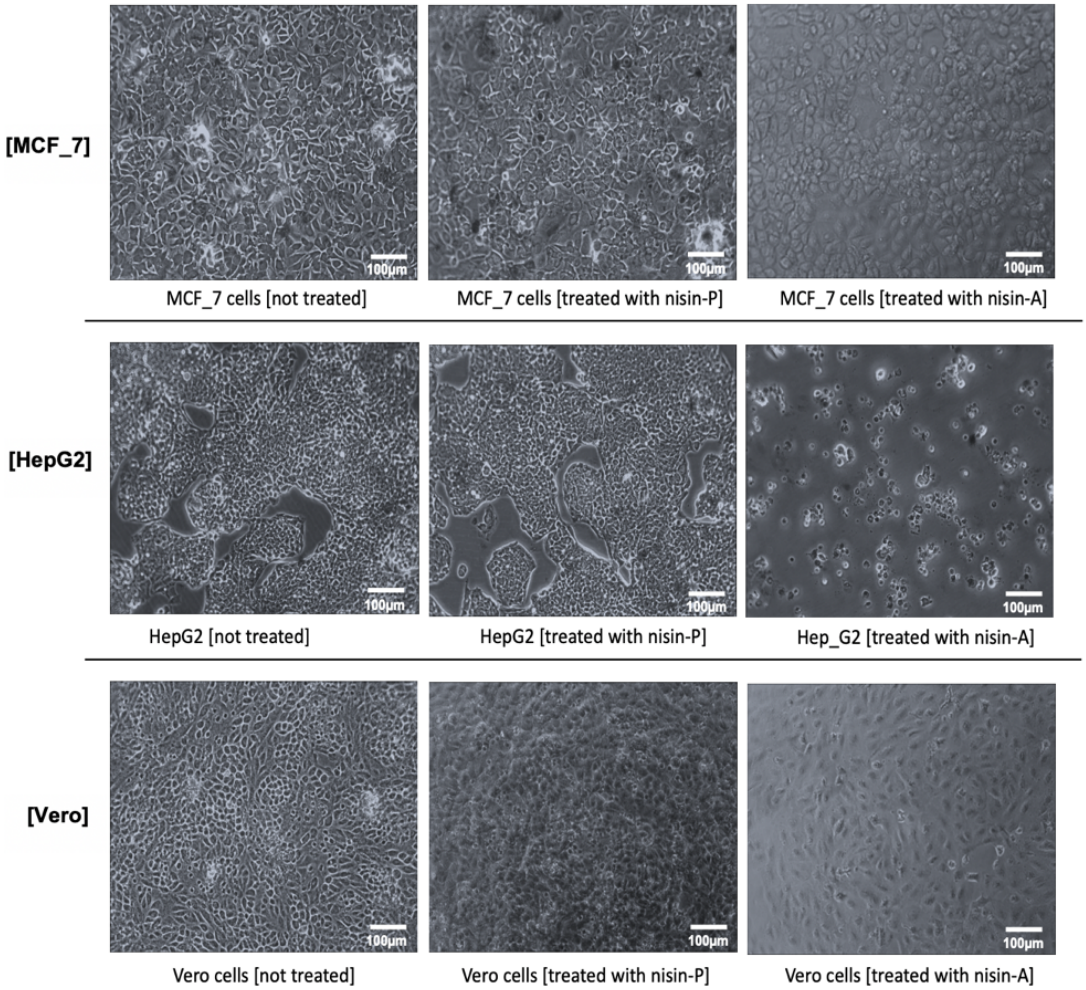

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775 Table 1

Nisin-p gene	Nisin-U gene [Accession No]	Putative function	Identity (%)
nspP	*nsup* [Q2QBT6]	Proteolytic cleavage of leader peptide	85.9
nspR	*nsuR* [Q2QBT5]	Lantibiotic regulation	86.2
nspK	*nsuK* [Q2QBT4]	Lantibiotic regulation	82.1
nspF	*nsuF* [Q2QBT3]	Immunity	85.7
nspE	*nsuE* [Q2QBT2]	Immunity	82.4
nspG	*nsuG* [Q2QBT1]	Immunity	81.2
nspA	*nsuA* [Q2QBT0]	Lantibiotic nisin U	90.9
nspB	*nsuB* [Q2QBS9]	Lantibiotic biosynthesis, dehydratase	92.4
nspT	*nsuT* [Q2QBS8]	Lantibiotic translocator/transporter	94.6
nspC	*nsuC* [Q2QBS7]	Thioether formation	89.2
nspI	*nsuI* [Q2QBS6]	Immunity	82.4

776 Table 2

Microorganism	Activity of nisin-P peptide (AU)
Gram-positive	
Bacillus cereus **(UoP strain-1.1)**	-
Bacillus subtilis **(UoP strain-1.1)**	-
Clostridium sporogenes **(UoP strain-1.1)**	++
Enterococcus faecalis **(NCTC 12697)**	++
Enterococcus faecalis **(VRE) (UoP strain-1.1)**	++
Listeria innocua **(UoP strain)**	-
Listeria monocytogenes **(UoP strain-1.1)**	-
Micrococcus luteus **(UoP strain-1.1)**	++++
Staphylococcus aureus **(NCTC 12493) [MRSA]**	+++
Staphylococcus aureus **(NCTC 12981) [MSSA]**	+++
Streptococcus agalactiae **(UoP strain-1.1)**	-
Streptococcus anginosus **(UoP strain-1.1)**	-
Streptococcus gallolyticus **(UoP strain-1.1)**	+++
Streptococcus pneumoniae**_DRF-clinical strain-1.1**	+++
Streptococcus pneumoniae**_DRF-clinical strain-1.3**	+++
Streptococcus pneumoniae**_DRF-clinical strain-1.4**	+++
Streptococcus pneumoniae**_DRF-clinical strain-1.5**	+++
Streptococcus pneumoniae**_DRF-clinical strain-1.6**	+++
Streptococcus pneumoniae **(NCTC 12695)**	+++
Streptococcus pneumoniae **(NCTC 12977) [PR]**	+++
Streptococcus pneumoniae **(UoP strain-1.1)**	++++
Streptococcus pyogenes **(UoP strain-1.1)**	++
Streptococcus uberis **(NCTC 3858)**	++
Gram-negative	
Enterobacter **species (UoP strain-1.1)**	-
Escherichia coli **(DH5$^{\alpha}$ strain)**	-
Escherichia coli **(NCTC 10418)**	-
Klebsiella pneumoniae **(NCTC 9633)**	-
Neisseria lactamica **(NCTC 10617)**	-
Pseudomonas aeruginosa **(NCTC 10662)**	-